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(54) **PREVENTING DESENSITIZATION OF RECEPTORS**

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(57) **ABSTRACT**

Desensitization of receptors that control disease is prevented by inhibiting G-protein receptor kinases. This has applicability, e.g., for patients with heart failure or on a left ventricular heart device or a heart pump after surgery or about to undergo surgery and at high risk for a cardiac event or on an opiate or addicted to opiate or with cystic fibrosis or rheumatoid arthritis.

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(12) **United States Patent**
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(54) **USE OF CENTRAL CANNABINOID RECEPTOR ANTAGONISTS FOR REGULATING APPETENCE**

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(58) **Field of Search** 514/406, 329, 514/231.5

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,707,497 A 11/1987 Cecchi et al. 514/647

FOREIGN PATENT DOCUMENTS

EP 0 656 354 6/1995

OTHER PUBLICATIONS

D. Shire et al., "Structural Features of the Central Cannabinoid CB1 Receptor Involved in the Binding of the Specific CB1 Antagonist SR 141716A", *J. Biol. Chem.*, 271 (12), 1996, pp. 6941–6946.

R. Pertwee et al., "AM640, A Competitive Cannabinoid Receptor Antagonist", *Life Sci.*, 56 (23/24), 1995, pp. 1941–1947.

M. Rinaldi–Carmona et al., "Biochemical and Pharmacological Characterisation of SR141716A, The First Potent and Selective Brain Cannabinoid Receptor Antagonist", *Life Sci.*, 56 (23/24), 1995, pp. 1949–1955.

F. Barth et al., "Cannabinoids: Receptors, Endogenous Ligands and A Newly Synthesized Antagonist", *NIDA Res. Monograph*, 162, 1996, pp. 52–53.

M. Rinaldi–Carmona et al., "Characterization and Distribution of Binding Sites for [^{3}H]–SR 141716A, A Selective Brain (CB1) Cannabinoid Receptor Antagonist, in Rodent Brain", *Life Sci.*, 58(15), 1996, pp. 1239–1247.

M. Rinaldi–Carmona et al., "SR141716A, A Potent and Selective Antagonist of the Brain Cannabinoid Receptor", *FEBS Letters*, 350 (2–3), 1994, pp. 240–244.

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(57) **ABSTRACT**

The invention relates to the use of a central cannabinoid receptor antagonist, by itself or in association with a compound for regulating metabolic disorders, especially a β_3-adrenergic receptor agonist, for the preparation of drugs useful in the treatment of appetency disorders.

13 Claims, No Drawings

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(54) Title: CRYSTALLINE SALTS OF 2-(4-{2-[2-HYDROXY-3-(2-THIOPHEN-2-YL-PHENOXY)-PROPYLAMINO]-2-METHYL-PROPYL}-PHENOXY)-NICOTINONITRILE

(57) Abstract: The present invention relates to crystalline salts of 2-(4-{2-[2-hydroxy-3-(2-thiophen-2-yl-phenoxy)-propylamino]-2-methyl-propyl}-phenoxy)-nicotinonitrile. The salts of the present invention, being β3 adrenergic receptor agonists, are capable of increasing lipolysis and energy expenditure in cells and, therefore, are useful, e.g., for treating type 2 diabetes and/or obesity.

CRYSTALLINE SALTS OF 2-(4-{2-[2-HYDROXY-3-(2-THIOPHEN-2-YL-PHENOXY)-PROPYLAMINO]-2-METHYL-PROPYL}-PHENOXY)-NICOTINONITRILE

This application claims the benefit of U.S. Serial No. 60/292,988, filed May 23, 2001.

Field of Invention

The present invention is in the field of medicine, particularly in the treatment of type 2 diabetes and obesity. More specifically, the present invention relates to a β_3 adrenergic receptor agonist useful in the treatment of type 2 diabetes and obesity.

Background of the Invention

The current preferred treatment for type 2, non-insulin dependent diabetes as well as obesity is diet and exercise, with a view toward weight reduction and improved insulin sensitivity. Patient compliance, however, is usually poor. The problem is compounded by the fact that there are currently no approved medications that adequately treat either type 2 diabetes or obesity.

One therapeutic opportunity that has recently been recognized involves the relationship between adrenergic receptor stimulation and anti-hyperglycemic effects. Compounds that act as β_3 receptor agonists have been shown to exhibit a marked effect on lipolysis, thermogenesis and serum glucose levels in animal models of type 2 (non-insulin dependent) diabetes.

The β_3 receptor, which is found in several types of human tissue including human fat tissue, has roughly 50% homology to the β_1 and β_2 receptor subtypes yet is considerably less abundant. Stimulation of the β_1 and β_2 receptors can cause adverse effects such as tachycardia, arrhythmia, or tremors. An agonist that is selective for the β_3 receptor over the β_1 and β_2 receptors is, therefore, more desirable for treating type 2 diabetes or obesity relative to a non-selective agonist.

However, recent studies have suggested the presence of an atypical beta receptor associated with atrial tachycardia in rats (*Br. J. of Pharmacol.*, 118:2085-2098, 1996). In other words, compounds that are not agonists of the β_1 and β_2 receptors can still modulate tachycardia through activation of a yet to be discovered β_4 or through some other unknown pathway.

A large number of publications have appeared in recent years reporting success in discovery of agents that stimulate the β_3 receptor. Despite these recent developments, there remains a need to develop a β_3 receptor agonist which has minimal or no agonist activity against the β_1 and β_2 receptors.

Summary of the Invention

The present invention is related to crystalline pharmaceutical acid addition salts of 2-(4-{2-[2-hydroxy-3-(2-thiophen-2-yl-phenoxy)-propylamino]-2-methyl-propyl}-phenoxy)-nicotinonitrile, hereafter referred to as SAM II.

More specifically, the present invention is related to crystalline non-solvated SAM II hemi-fumarate, hereafter referred to as the "hemi-fumarate F-I"

In addition, the present invention is related to crystalline SAM II hemi-fumarate hemi-hydrate, hereafter referred to as the "hemi-fumarate hemi-hydrate".

In addition, the present invention is related to non-solvated crystalline SAM II benzoate, hereafter referred to as the "benzoate".

In addition, the present invention is related to non-solvated crystalline SAM II (R)-mandelate, hereafter referred to as the "(R)-mandelate".

Moreover, the present invention is related to non-solvated crystalline SAM II salicylate, hereafter referred to as the "salicylate".

The present invention also relates to pharmaceutical formulations containing a crystalline salt of the present invention and a pharmaceutical carrier. In another embodiment, the pharmaceutical formulations of the present invention may be adapted for use in treating type 2 diabetes and/or obesity and/or for agonizing the β_3 receptor.

The present invention also relates to methods for treating type 2 diabetes and/or obesity, as well as a method for agonizing the β_3 receptor, which comprises administering to a patient in need thereof an effective amount of a salt of the present invention.

In addition, the present invention relates to the salts of the present invention for use in treating type 2 diabetes and/or obesity as well as the salts of the present invention for use in agonizing the β_3 receptor. The present invention is further related to the use of the salts of the present invention for the manufacture of a medicament for treating type 2 diabetes and/or obesity as well as for agonizing the β_3 receptor.

Brief Description of the Figures

Figure 1 is a representative XRD pattern for the hemi-fumarate F-I.

Figure 2 is a representative XRD pattern for the hemi-fumarate hemi-hydrate.

Figure 3 is a representative XRD pattern for the benzoate.

Figure 4 is a representative XRD pattern for the (R)-mandalate.

Figure 5 is a representative XRD pattern for the salicylate.

Detailed Description of the Invention

Characterization

Various methods, including differential thermal/thermogravimetric analysis (DT/TGA), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), X-ray powder diffraction (XRD) and ^{13}C solid state nuclear magnetic resonance (SSNMR) were used to characterize the salts of the present invention. DT/TGA is a combined system that allows for simultaneous measurement of the amount and rate of weight change (TGA) and the temperatures of endothermic and exothermic transitions (DTA). TGA is most commonly used to study desolvation processes and quantatively determine the total volatile content of a solid. DSC is a technique that is often used to screen compounds for polymorphism because the temperatures(s) at which a physical change in a material occurs is usually characteristic of that material. DSC is often used to compliment TGA analysis in screening compounds for physical changes upon controlled heating. XRD is a technique that detects long-range order in a crystalline material.

DT/TGA was carried out on a TA simultaneous TG/DTA unit (model SDT2960). Samples were heated in open aluminum pans from 25 to 300 °C at 10 °C/min with a nitrogen purge of 150 mL/min. The temperature was calibrated with indium. The weight calibration was performed with manufacturer-supplied standards and verified against sodium tartrate desolvation.

DT/TGA analysis of the hemi-fumarate F-I showed no weight loss prior to the onset of melting at ~170°C, as expected for a non-solvated crystal form.

DT/TGA analysis of the hemi-fumarate hemi-hydrate showed a weight loss of 1.6% indicating that a stoichiometric 0.5 mole hydrate was present. An endotherm at ~147 °C in the DTA trace represents the melt of the hemi-fumarate hemi-hydrate desolvate.

DSC analysis was also carried out on a TA Instruments Model 2910 DSC and/or Model 2920 MDSC: Model 2950 TGA and TGA analysis was carried out on a Model 2950 TGA . DSC samples were heated in aluminum pans from ambient to 300°C at 5°C/minute with a nitrogen purge. TGA samples were heated in a platinum pan from ambient to 300°C with nitrogen purge.

TGA analysis of the benzoate showed no weight loss (consistent with a non-solvated crystalline form) and a melting endotherm was observed at 149°C by DSC.

TGA analysis of the (R)-mandelate shows no weight loss (consistent with a non-solvated crystalline form) and the DSC trace shows only a single sharp melting endotherm at ~138°C.

TGA analysis of the salicylate shows minimal weight loss suggesting that the material is non-solvated, while the DSC trace shows a melting endotherm at ~125°C.

X-ray powder diffraction patterns were obtained on a Siemens D5000 X-ray powder diffractometer which was equipped with a CuKα source (λ = 1.54056) operated at 50 kV and 40 mA with a Kevex solid state Si(Li) detector. The samples were scanned from 4 to 35° in 2θ at 3.0 seconds per step size of 0.02 ° with 1 mm divergence and receiving slits and a 0.1 mm detector slit. The dry powders were packed into recessed top-loading sample holders and a smooth surface was obtained using a glass slide.

Representative XRD traces of the hemi-fumarate F-I, the hemi-fumarate hemi-hydrate, benzoate, (R)-mandelate and salicylate are shown in Figures 1-5, respectively. The XRD patterns feature sharp peaks and a flat baseline, indicative of highly crystalline materials. The angular peak positions in 2θ and corresponding I/I_0 data for all peaks with intensities equal to or greater than 10% of the largest peak for the hemi-fumarate F-I, benzoate and salicylate are tabulated in Tables 1, 3 and 5, respectively. The angular peak positions in 2θ and corresponding I/I_0 data for all peaks with intensities equal to or

greater than 5% of the largest peak for the hemi-fumarate hemi-hydrate and the (R)-mandelate are tabulated in Tables 2 and 4, respectively. All data in Tables 1-5 are expressed with an accuracy of ± 0.1° in 2θ .

Table 1

Angle 2θ	I/I_o (%)	Angle 2θ	I/I_o (%)
9.8	13.1	20.7	18.3
11.4	50.0	21.5	10.5
12.6	10.5	21.8	18.2
15.6	19.2	22.6	16.3
17.6	63.6	23.2	21.8
17.9	39.9	23.3	24.0
18.6	35.7	24.8	21.0
18.8	24.4	25.1	12.6
19.4	21.6	27.1	25.4
20.3	100	30.2	17.5

Table 2

Angle 2θ	I/I_o (%)	Angle 2θ	I/I_o (%)
4.2	7.9	18.6	37.5
7.8	6.4	20.6	13.1
8.4	13.6	21.3	68.7
9.9	28.9	22.9	11.9
11.4	100	23.3	13.9
12.7	51.2	23.8	14.5
15.2	19.2	25.3	6.3
15.3	13.3	26.3	5.5
16.7	6.7	27.4	11.9
17.4	7.1		

Table 3

Angle 2θ	I/I_o (%)	Angle 2θ	I/I_o (%)
5.6	29.0	26.0	41.2
7.1	36.2	26.5	14.9
8.3	26.9	26.7	12.7
8.6	100	29.8	13.5
13.4	14.7	18.7	56.5
13.7	11.8	19.3	68.3
14.9	68.0	19.9	16.9
16.1	11.6	20.4	67.2
16.7	39.2	21.5	14.8
16.8	36.2	21.8	34.6
17.2	55.4	22.2	83.5
17.6	50.9	22.4	40.2
17.8	24.8	23.3	48.6
18.0	10.3	24.2	20.1
18.4	33.4	24.7	13.0
25.6	18.3		

Table 4

Angle 2θ	I/I_o (%)	Angle 2θ	I/I_o (%)
4.7	100	18.6	7.6
13.2	10.2	20.0	9.0
13.4	5.6	20.5	7.8
14.5	5.9	21.1	21.7
15.3	5.2	21.8	15.1
16.9	10.0	22.3	5.2
18.2	8.3	23.5	5.7

Table 5

Angle 2θ	I/I_o (%)	Angle 2θ	I/I_o (%)
6.9	33.8	19.0	32.1
8.2	28.3	19.3	27.4
8.8	29.4	19.6	21.3
13.7	21.0	20.1	11.4
14.6	100	21.8	23.3
16.6	20.6	22.4	22.1
16.9	39.0	22.6	68.5
17.7	19.6	23.5	10.7
18.0	83.8	24.9	23.5

It is well known in the crystallography art that, for any given crystal form, the relative intensities of the diffraction peaks may vary due to preferred orientation resulting from factors such as crystal morphology and habit. Where the effects of preferred orientation are present, peak intensities are altered, but the characteristic peak positions of the polymorph are unchanged. See, *e.g.*, The United States Pharmacopeia #23, National Formulary #18, pages 1843-1844, 1995. Furthermore, it is also well known in the crystallography art that, for any given crystal form, the angular peak positions may vary slightly. For example, peak positions can shift due to a variation in the temperature at which a sample is analyzed, sample displacement, or the presence or absence of an internal standard. In the present case, a peak position variability of ± 0.1° in 2θ will take into account these potential variations without hindering the unequivocal identification of the crystalline salts of the present invention.

A well-known and accepted method for searching crystal forms in the literature is the "Fink" method. The Fink method, in general, uses the four most intense lines for the initial search followed by the next four most intense lines.

In general accord with the Fink method, based on peak intensities as well as peak position, the hemi-fumarate F-I may be identified by the presence of peaks at 11.4 ± 0.1, 17.6 ± 0.1, 17.9 ± 0.1 and 20.3 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056). The presence of the hemi-fumarate F-I may be further

verified by peaks at 18.6 ± 0.1, 18.8 ± 0.1, 19.4 ± 0.1 and 27.1 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

In general accord with the Fink method, based on peak intensities as well as peak position, the hemi-fumarate hemi-hydrate may be identified by the presence of peaks at 11.4 ± 0.1, 12.7 ± 0.1, 18.6 ± 0.1 and 21.3 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056). The presence of the hemi-fumarate hemi-hydrate may be further verified by peaks at 8.4 ± 0.1, 9.9 ± 0.1, 15.2 ± 0.1 and 23.8 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056). Peaks at 4.2 ± 0.1 and 7.8 ± 0.1° in 2θ are also highly indicative of the presence of the hemi-fumarate hemi-hydrate.

In accord with the Fink method, based on peak intensities as well as peak position, the benzoate may be identified by the presence of peaks at 8.6 ± 0.1, 14.9 ± 0.1, 19.3 ± 0.1 and 22.2 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056). The presence of the benzoate may be further verified by peaks at 17.2 ± 0.1, 17.6 ± 0.1, 18.7 ± 0.1 and 20.4 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

In accord with the Fink method, based on peak intensities as well as peak position, the (R)-mandelate may be identified by the presence of peaks at 4.7 ± 0.1, 13.2 ± 0.1, 21.1 ± 0.1 and 21.8 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056). The presence of the (R)-mandelate may be further verified by peaks at 16.9 ± 0.1, 18.2 ± 0.1, 18.6 ± 0.1 and 20.0 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

In accord with the Fink method, based on peak intensities as well as peak position, the salicylate may be identified by the presence of peaks at 14.6 ± 0.1, 16.9 ± 0.1, 18.0 ± 0.1 and 22.6 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056). The presence of the salicylate may be further verified by peaks at 6.9 ± 0.1, 8.2 ± 0.1, 8.8 ± 0.1 and 19.0 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

^{13}C SSNMR analysis was performed with a Varian Unity Inova 400 MHz spectrometer operating at a carbon frequency of 100.573 MHz, using high-power proton decoupling, cross polarization (CP) and magic angle spinning (MAS) at ~7.0 kHz.

Acquisition parameters were as follows: 90° proton r.f. pulse width 4.0 μs, contact time 2.5 ms, pulse repetition time 5 s, spectral width 50 kHz, and acquisition time 50 ms. Chemical shifts, expressed as parts per million, were referenced to the methyl group of hexamethylbenzene (δ = 17.3 ppm) by sample replacement. The magic angle was adjusted by optimizing the sidebands of the ^{79}Br signal of KBr as described by Frye and Maciel (Frye J. S. and Maciel G. E., *J. Magn. Reson.*, **1982**, *48*, 125).

The SSNMR spectrum for the hemi-fumarate F-I comprises isotropic peaks at the following chemical shifts: 19.3, 20.5, 24.5, 26.7, 40.6, 44.9, 59.5, 65.1, 66.4, 70.8, 96.5, 97.4, 113.3, 115.0, 118.4, 121.8, 123.7, 128.0, 131.3, 132.7, 133.9, 137.1, 140.4, 145.4, 150.5, 152.2, 154.8, 164.5, 172.4, 174.1 ppm.

The SSNMR spectrum for the hemi-fumarate hemi-hydrate comprises isotropic peaks at the following chemical shifts: 20.6, 22.0, 23.1, 24.3, 45.2, 59.2, 60.0, 65.2, 66.4, 70.2, 74.1, 98.4, 114.7, 116.5, 119.2, 121.8, 122.6, 127.2, 131.0, 132.7, 134.3, 134.8, 137.6, 139.8, 143.9, 151.9, 154.4, 155.4, 163.6, 173.5, 177.4 ppm.

Synthesis

Potentiometric titrations of SAM II were performed using 0.1 M aqueous KCl medium and the measured pK_a was determined to be 8.51 +/- 0.09. Therefore, the acid addition crystalline salts of the present invention are preferably those formed from reaction of SAM II with pharmaceutical acids that have pK_a's of at least 2 units lower than the protonated amine (in order to ensure a complete acid-base reaction).

Hemi-Fumarate F-I

The hemi-fumarate F-I may be crystallized from various organic solvents, including methanol, ethanol, isopropyl alcohol, n-propanol, acetonitrile, dimethylformamide, ethyl acetate, toluene and mixtures thereof. The hemi-fumarate F-I may also be crystallized from aqueous-organic solvent mixtures, including methanol-water, ethanol-water, isopropyl alcohol-water, acetonitrile-water, and acetone-water, when ≤50% water is present upon crystallization.

The hemi-fumarate F-I may also be prepared by recrystallizing the hemi-fumarate hemi-hydrate from the above-mentioned solvent systems.

Hemi-fumarate Hemi-hydrate

The hemi-fumarate hemi-hydrate may be prepared by recrystallizing the hemi-fumarate F-I from tetrahydrofuran-water, dimethylsulfoxide-acetone/water or dimethylformamide-ethanol/water at ambient temperature when >50% water is present at the time of nucleation. In the absence of hemi-fumarate F-I seeds, the hemi-fumarate hemi-hydrate may be prepared from ethanol and ethanol/ethyl acetate mixtures.

Benzoate

The benzoate may be isolated from single and mixed polar to moderately polar solvents (e.g., isopropyl alcohol, isopropyl alcohol-water, acetonitrile, ethanol-ethyl acetate, ethyl acetate, methyl ethyl ketone) when the temperature gradient method is employed and crystallization occurs between 25 and 50°C. For example, the benzoate may be obtained by crystallization in ethyl acetate at 50°C or a prolonged reslurry at 50°C (at a dilution which gives some solubility to the mixture). Preferred crystallization solvent systems include 9:1 ethyl acetate:ethanol, isopropyl alcohol, and acetonitrile. The benzoate salt crystallized as birefringent rods from 95% ethanol and as fine hair-like needles from the other solvents after they were allowed to evaporate.

(*R*)-Mandelate

The (*R*)-mandelate salt of the present invention may be prepared from the isomorphic ethyl acetate, isopropyl acetate, acetone, methyl ethyl ketone, ethanol and isopropyl alcohol solvates of the (*R*)-mandelate by slurrying any of said solvates in water.

Crystallization solvent systems that will produce the non-solvated form directly, via use of an (*R*)-mandelate seed, are acetone; 9% acetone/ethyl acetate (11 mL/g, 92% yield); 20% acetone/ethyl acetate (12.5 mL/g, 86% yield); and 20% isopropyl alcohol/ethyl acetate (12.5 mL/g, 92% yield).

Salicylate

The salicylate may be crystallized from 96:4 ethanol (denatured with toluene):water. Preferably, the SAM II free base starting material contains <6% total related substances (TRS), *e.g.*, the SAM II prepared in Preparation 2 below.

Hemi-fumarate F-I vs. Hemi-fumarate Hemi-hydrate

The physical stability of the hemi-fumarate F-I and the hemi-fumarate hemi-hydrate were evaluated in solution and in the solid-state as a function of temperature and relative humidity. The hemi-fumarate F-I, which melts at 176°C, is thermally more stable than the hemi-fumarate hemi-hydrate, which de-solvates above ambient temperature and subsequently melts at ~150°C. The hemi-fumarate F-I is non-hygroscopic and is thermodynamically more stable than the hemi-fumarate hemi-hydrate in all aqueous and organic media tested.

In the solid state, the hemi-fumarate hemi-hydrate is stable from 10 to 95% RH at ambient temperature but can be reversibly de-solvated above ambient temperature or upon exposure to low relative humidity at ambient temperature (~25°C).

The hemi-fumarate F-I's superior stability relative to the hemi-fumarate hemi-hydrate was demonstrated by suspending the hemi-fumarate hemi-hydrate in a solvent in which the hemi-fumarate F-I is slightly soluble and stirring in the presence of seed crystals of the hemi-fumarate F-I. Complete conversion to the hemi-fumarate F-I was observed (confirmed by X-ray diffraction and melting point) after stirring in ethanol (denatured with toluene) for 18-hours at room temperature or after 1 hour at 78°C.

The aqueous solubility for both forms varies as a function of pH, increasing with decreasing pH. The hemi-fumarate hemi-hydrate has nearly twice the solubility of the hemi-fumarate F-I at several pH's in aqueous solution. However, the solubility of both are nearly comparable in simulated intestinal fluid (fed), and enhanced relative to that in simulated intestinal fluid (fasted), suggesting that the compounds may have significant dissolution in the intestinal tract. In most organic solvents tested, the hemi-fumarate F-I is significantly less soluble than the hemi-fumarate hemi-hydrate.

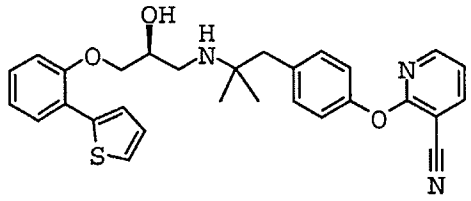
The hemi-fumarate F-I gave lower exposures in both F344 rats and Cynomolgus monkeys than either the hemi-fumarate hemi-hydrate or the HCl salt. However, the exposures are acceptable to permit development of the hemi-fumarate F-I.

The hemi-fumarate F-I also has acceptable processing characteristics (granular particules which filter rapidly) and an acceptable impurity rejection (though the impurity rejection is not quite as good as the hemi-fumarate hemi-hydrate).

The following examples illustrate specific procedures for preparing the crystalline salts of the present invention.

Preparations and Examples

Preparation 1: Synthesis of SAM II



A mixture of 2-(thien-2-yl)phenol (*J. Heterocycl. Chem.*, 22(6):1667-9, 1985; 1 equivalent), (2S)-glycidyl 3-nitrobenzenesulfonate (1.2 equivalent), potassium carbonate (1.2 equivalent) and acetone (8.6 ml/mol of phenol) are refluxed for 16 hours, cooled to room temperature and the solids are removed via filtration. The filtrate is concentrated and the crude product purified on silica gel (40% ethyl acetate/hexane) to give the desired epoxide.

4-(2-Amino-2-methylpropyl)phenol (50.8 g, 225 mmol), 2-chloro-3-cyanopyridine (30.8 g, 222 mmol), potassium carbonate (77.7 g, 562 mmol, powdered), N,N-dimethylacetamide (609 ml), and isooctane (122 ml) are combined and heated to reflux. The water formed during the reaction is removed azeotropically via a Dean-Stark trap. After about 1-2 hours the reaction is complete. The slurry is cooled to 30°C and filtered. The filter cake is washed with N,N-dimethylacetamide (250 ml) and the combined organic fractions are concentrated by rotary evaporation at 80°C. The resulting dark green oil is dissolved in dichloromethane (580 ml), and washed with water (160 ml). The phases are separated and the organic phase washed with water (250 ml). Water (1 L) is added to the organic phase and the pH adjusted to 1 with 12N aqueous hydrochloric acid (about 25 ml). The phases are separated and the acidic aqueous layer is washed with dichloromethane (250 ml). Dichloromethane (1 L) is added to the acidic aqueous phase and the pH is adjusted to 12-13 with 5N aqueous sodium hydroxide. The phases are

separated and the organic phase is dried over sodium sulfate. After filtration the solution is concentrated to give 53 g of the desired amine (88%).

A stirred mixture of the epoxide (1 equivalent) and the amine (1-2 equivalents) in ethanol, methanol, n-butanol or t-butanol is heated at 70-80°C for 2-72 hours. The solvent is evaporated to dryness to give a crude oil that is optionally diluted with methanol or ethanol and passed over a cation exchange column (eluting the free base product with 1N methanolic ammonia) before further purification.

The final product may be further purified by flash or radial chromatography. Typical chromatography conditions include: a) using a variable mixture of 25:5:1 chloroform/methanol/ammonium hydroxide and 9:1 chloroform/methanol; b) a variable mixture of 90:10:1 CH_2Cl_2/ethanolic NH_3 gradient; c) dichloromethane/6-12% methanol, 0.15-0.35M ammonia in dichloromethane gradient; d) methylene chloride with a step gradient to 2-8% methanol; e) chloroform/2.0M ammonia in methanol, from 0-10% to 6-20% gradient elution or f) isocratic 6-8% 2M ammonia in methanol: 92-94% dichloromethane.

Preparation 2: Alternative Synthesis of SAM II

4-(2-amino-2-methylpropyl)phenol acetic acid salt (45.06 g, 200 mmol) is added to water (350 mL) and stirred at 30°C until the solid dissolves. Sodium hydroxide (5N, 41 mL, 205 mmol) is added over 5 minutes and rinsed with water (10 mL). The resulting slurry is stirred for 1 hour at 25°C followed by 45 minutes at <10°C. The product is filtered and washed with cold water (2 x 25 mL). The product is dried in a vacuum oven (50°C, 5 mmHg, 18 hours) to give 30.96 g (93.7%) of 4-(2-amino-2-methylpropyl)phenol.

Ethanol (750 mL, 2B-3), (2*S*)-1-(2-(thien-2-yl)phenyloxy)-2,3-epoxypropane (75.0 g, 323 mmol), 4-(2-amino-2-methylpropyl)phenol (64.0 g, 387 mmol) and glacial acetic acid (485 mg, 8.07 mmol) are combined. The resulting yellow solution is stirred at 75-80°C for 18 hours until none of the epoxy starting material remained by HPLC then dimethylsulfoxide (246 mL) is added. Terephthalic acid (24.7 g, 149 mmol) is dissolved in dimethylsulfoxide (246 mL) at 60°C and then added rapidly, rinsing with 60°C dimethylsulfoxide (123 mL). The solution is seeded with (2S)-3-{[2-(4-hydroxyphenyl)-*tert*-butyl]amino}-1-(2-(2-thienyl)phenoxy)propan-2-ol terephthalate (2:1) and stirred at

80°C for 1 hour. The white mixture is cooled to 60°C over 30 minutes and stirred for 30 minutes, cooled to 40°C over 30 minutes and stirred for 30 minutes, cooled to 20-25 °C over 30 minutes and stirred 1 hour, then filtered. The wet cake is reslurried three times in ethanol (750 mL, 2B-3) for 30 minutes then filtered. After the final filtration the cake is rinsed with ethanol (300 mL, 2B-3). After vacuum drying at 50°C/5 Torr for 12 hours, 121.2 g (77.8%) of (2S)-3-{[2-(4-Hydroxyphenyl)-*tert*-butyl]amino}-1-(2-(2-thienyl)phenoxy)propan-2-ol terephthalate (2:1) is obtained as a white solid, mp 160-162°C.

(2*S*)-3-{[2-(4-hydroxyphenyl)-*tert*-butyl]amino}-1-(2-(2-thienyl)phenoxy)propan-2-ol terephthalate (2:1 salt) (20.0 g, 41.4 mmol), 2-chloro-3-cyanopyridine (5.97 g, 43.1 mmol), potassium carbonate (13.73 g, 99.4 mmol) and DMSO (80 mL) are mixed. The resulting slurry is heated with stirring at 85°C until the reaction is complete (approximately 7 hours). The slurry is cooled to 30°C, Hyflow® (8 g) is added, and the mixture is stirred for 10 minutes. Ethyl acetate (140 mL) is added and the mixture is stirred for approximately 15 minutes. The mixture is filtered and the filter cake is rinsed with ethyl acetate (60 mL). The filtrate is washed sequentially with 5% NaCl solution (200 mL), 5% $NaHCO_3$ solution (200 mL), and 5% NaCl solution (2 x 200 mL). The organic layer is concentrated by rotary evaporation to give SAM II.

Preparation 3: Preparation of SAM II (R)-Mandelate Ethyl Acetate Solvate

SAM II is suspended in ethyl acetate and one molar equivalent R-mandelic acid is added as a powder. The slurry is heated to effect dissolution of the solids, then gradually cooled to room temperature. The solid precipitate is isolated by vacuum filtration and rinsed with ethyl acetate to give the title compound.

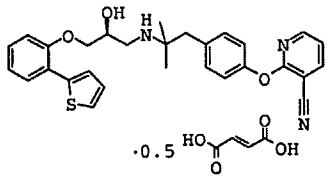
Preparation 4: Preparation of SAM II (R)-Mandelate Ethanolic Solvate

SAM II (253 mg) is suspended in 5 mL of ethanol denaturated with toluene and one molar equivalent R-mandelic acid (76 mg) is added as a powder. The slurry is heated to effect dissolution of the solids, then gradually cooled to room temperature and seeded with crystals of the ethyl acetate solvate of the (R)-mandelate salt. Considerable

precipitation is observed within minutes and the slurry is maintained overnight. The thick crystal slurry is diluted with 2 mL of ethanol denatured with toluene, isolated by vacuum filtration and rinsed with 3-5 mL of ethanol denatured with toluene. Yield = 250 mg of the ethanol solvate (TGA weight loss = 3.8%).

Example 1

Hemi-Fumarate F-I



SAM II (254 mg) is dissolved in 4 mL of absolute anhydrous ethanol at reflux (~76°C). Fumaric acid (0.5 molar equivalent, 32 mg) is added as a powder to the refluxing solution and rinsed in with 1 mL of absolute anhydrous ethanol. Solids are observed to rapidly precipitate from the refluxing solution. The crystal slurry is maintained at reflux for approximately 30 minutes, then allowed to cool to room temperature and resonate overnight. The solid product is isolated by vacuum filtration and washed with 3-5 mL of absolute anhydrous ethanol. Yield = 253 mg.

Example 1(a)

Alternative Preparation of the Hemi-fumarate F-I

The organic layer that is concentrated by rotary evaporation to give SAM II as described in Preparation 2 is concentrated to approximately 80 mL of solution and is diluted with ethanol (denatured with toluene, 120 mL). The solution is heated to 65°C with stirring and fumaric acid (2.16 g, 18.6 mmol) in ethanol (denatured with toluene, 200 mL) is added. The solution is seeded and stirred at 65°C for 20 minutes. The resulting slurry is slowly cooled to room temperature over approximately 2.25 hours, then cooled to approximately 0°C for 30 minutes. The product is filtered and washed with cold 2B-3 ethanol (60 mL). The product is dried in a vacuum oven (50 °C, 5 mmHg, 18 h) to give 19.52 g (86.1%) of the title compound as a white solid, mp 172.5-174°C.

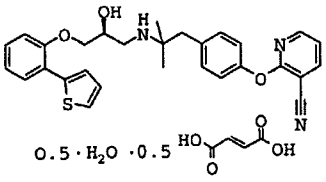
Example 1(b)

Alternative Preparation of the Hemi-fumarate F-I

The hemifumarate hemihydrate (100 mg) is suspended in 3 mL of absolute anhydrous ethanol, and the slurry is heated to reflux. Incomplete dissolution is observed, so an additional 2 mL absolute anhydrous ethanol is added. The slurry is maintained at reflux for about 15 minutes and then allowed to cool to room temperature. The solids are isolated by vacuum filtration and washed with a few mL of absolute anhydrous ethanol. Yield = 87 mg.

Example 2

Hemi-Fumarate Hemi-Hydrate



SAM II (250 mg) and 0.5 molar equivalents (29 mg) of fumaric acid are suspended in 5 mL of ethanol denatured with toluene and the mixture is heated mildly to effect dissolution. After approximately five minutes, the solution begins to precipitate. The temperature of the crystal slurry is maintained at the crystallization temperature (56-57°C) for about one hour. The heat source is then turned off and the slurry is allowed to cool with stirring overnight. Ethanol denatured with toluene (2 mL) is added and the solids are isolated by vacuum filtration. The filter cake is washed with ethanol denatured with toluene (5 mL) and air dried to give 230 mg of the title compound. mp = 147-149°C (measured by differential scanning calorimetry (DSC) with a scan rate of 10°C/minute).

Example 2(a)

Alternative Synthesis of the Hemi-Fumarate Hemi-Hydrate

The hemi-fumarate hemi-hydrate is prepared by dissolving the hemi-fumarate F-I (101 mg) in 1:1 v/v tetrahydrofuran-water (3.5 mL) and adding water (10 mL) dropwise at ambient temperature. The solution becomes milky with minimal water addition and a solid precipitate is observed after addition of ~5 mL water. The solid product is immediately isolated by vacuum filtration and washed with water. Yield = 82 mg of hairs/rods.

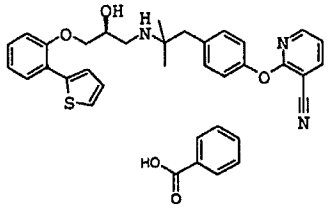
Example 2(b)

Alternative Synthesis of the Hemi-Fumarate Hemi-Hydrate

The hemi-fumarate hemi-hydrate is prepared by dissolving the hemifumarate F-I (101 mg) in 1:1 v/v dimethylsulfoxide-acetone (4 mL) and adding water (20 mL) dropwise at ambient temperature. Approximately 9 mL water is required to effect precipitation. The solids are immediately isolated by vacuum filtration and washed with water. Yield = 92 mg, clusters of rods.

Example 3

The Benzoate



SAM II (57.7 mg) is dissolved in 2.5 mL of absolute ethanol and the solution is stirred at room temperature. To the stirred solution is added benzoic acid (1 equivalent, 14.1 mg) dissolved in 200 microliters of methanol. The resulting mixture is stirred at room temperature for 3.5 to 4 hours. Precipitation occurs in approximately 30-60 minutes. The precipitate is isolated by vacuum filtration and the filter cake is collected

and air-dried overnight. mp = 148-150°C (measured by DSC with a scan rate of 5°C/minute).

Example 3(a)

Alternative Preparation of the Benzoate

Benzoic acid (3.66 g, 30 mmol) is added to a 50°C solution of SAM II (15.0 g, 30 mmol) in ethyl acetate (300 mL, 20 mL/g freebase). The homogeneous solution is seeded and after approximately 5 minutes, nucleation occurred. The mixture is held at 50°C for 4 hours during the precipitation. After cooling to room temperature (27°C), the mixture is filtered and the solid is washed with ethyl acetate (45 mL). The product is air-dried for 30 minutes to give 11.6 g (62% yield) of white, hair-like needles. The solid is vacuum-dried at 50°C/5 Torr with no additional loss of weight.

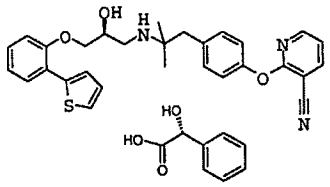
Example 3(b)

Alternative Preparation of the Benzoate

Solid benzoic acid (1.22 g, 10.0 mmol) is added to an approximately 60°C solution of SAM II (5.00 g, 90.7% pure, 9.07 mmol) in 90:10 ethyl acetate:ethanol (100 mL). The solution is seeded with a small amount of the title compound. The solution is allowed to cool to approximately 47°C and stir for 2-3 hours while the product precipitates. The slurry is cooled to room temperature and filtered. The filter cake is washed with cold ethyl acetate (20 mL) and dried in a vacuum oven overnight at 50°C to give 4.05 g (71.8% yield, 99.1% purity) of the title compound as a white solid, mp 149.8°C (by DSC).

Example 4

The (R)-Mandelate



SAM II (200 mg) is dissolved in 1 mL of acetone and the solution is stirred at room temperature. To the stirred solution is added R-mandelic acid (1 equivalent, 61 mg) in acetone (1 ml). The resulting mixture is stirred at room temperature and the precipitate is isolated by vacuum filtration. The filter cake is collected and air-dried overnight. mp = 138-140 °C (measured by DSC with a scan rate of 5°C/minute).

Example 4(a)

Alternative Preparation of the (R)-Mandelate

A solution of (*R*)-mandelic acid (6.09 g, 40.0 mmol) in acetone (30 mL) is added to a solution of SAM II (20.0 g, 40.0 mmol) in acetone (100 mL, 5.0 mL/g freebase) at 57°C. The homogeneous solution is heated at reflux for 5 minutes and then slowly cooled. The solution is seeded at 44.3°C. Crystallization began at 41°C. After cooling to room temperature, the mixture is filtered and the filter cake is washed with room temperature acetone (10 mL). The solid is air-dried for 15 minutes and vacuum-dried overnight at 50°C/5 Torr to afford 22.09 g (84.7% yield) of white solid.

The solid and filtrate are recombined, diluted with acetone to a total volume of 130 mL, heated to 56°C, and the solution is filtered to remove a few fine particles. The solution is cooled very slowly while seeding repeatedly with at 50°C, 48°C, and 47°C, at which point precipitation began to occur. The mixture is held at 47°C for 45 minutes, held at 45°C for 30 minutes, and is then cooled at a rate of 2.5°C every 15 minutes until reaching 25°C. The mixture is filtered but the solid is not washed. Instead, the solid is air-dried in the filter overnight and is then vacuum-dried at 50°C/5 Torr to give 17.1 g of white crystalline product (85% yield, 0.2% TRS).

Example 4(b)

Alternative Preparation of the (R)-Mandelate

To a solution of R-(-)-mandelic acid (0.3 g, 2.00 mmol) in ethyl acetate (3 mL) at temperature 50°C is added SAM II (1 g, 2 mmol) dissolved in ethyl acetate (4 mL). The flask is rinsed with ethyl acetate (3 mL). Acetone (1 mL) is added. The solution is heated to a reflux, seeded, then cooled in 5 °C increments. Once crystal formation starts, the solution is held at that temperature for one hour, then cooled at a rate of 5°C every 30 minutes until at 2° C. The solid is collected by vacuum filtration at 40°C under vacuum for two days.

Alternatively, Experiment 4(b) may be performed with an initial reaction temperature of 40°C. Under these conditions, 2.5 ml (instead of 1 mL) of acetone is used.

Alternatively, Experiment 4(b) may be performed with an initial reaction temperature of 65°C. Under these conditions, isopropyl alcohol is used in place of ethyl acetate and 2.5 ml (instead of 1 mL) of acetone is used.

Alternatively, Experiment 4(b) may be performed using acetonitrile in place of ethyl acetate. Under these conditions, the addition of acetone is not necessary.

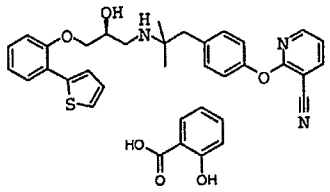
Example 4(c)

Alternative Preparation of the (R)-Mandelate

The compound of Preparation 4 (200 mg) is slurried in 5 mL of water at ambient temperature for 28 hours. The solids are then isolated by vacuum filtration, washed with 2-3 mL water, dried under an air stream for 30 minutes and then vacuum dried at 55°C for about 2 hours. m.p. = 138°C.

Example 5

The Salicylate



SAM II (106 mg) is dissolved in 1 mL of ethyl acetate and the solution is stirred at room temperature. To the stirred solution is added salicylic acid (1 equivalent, 29 mg) in 150 microliters of methanol. The resulting mixture is stirred at room temperature and then heated up 50°C. Hexane is added to the mixture at elevated temperature until cloud point (approximately 1 ml ethyl acetate:1 ml of hexane). The slurry is allowed to slowly cool to room temperature. The precipitate is isolated by vacuum filtration and the filter cake is collected and air dried overnight. mp = 124 °C (measured by DSC with a scan rate of 5°C/minute).

Example 5(a)

Alternative Preparation of the Salicylate

A solution of salicylic acid (4.15 g, 30 mmol) in 96:4 ethanol/water (25 mL) is added to a solution of SAM II (15.0 g, 30 mmol) in 96:4 ethanol (denatured with toluene)/water (75 mL, 5.0 mL/g freebase) at 75 °C. The homogeneous solution is cooled slowly and seeded every 5°C. The seeds remained undissolved at 50-55 °C and nucleation occurred at 50°C. The temperature is held at 45-50 °C for 1 hour while the product precipitates. The mixture became thick and difficult to stir magnetically so an additional 25 mL of warm 96:4 ethanol denatured with toluene/water is added to facilitate stirring. After 15 min, the heating mantle is removed and the mixture is allowed to cool slowly to approximately 30°C. The mixture is filtered, the filter cake is washed with ethanol denatured with toluene (15 mL), and the solid is air-dried to give 14.97 g (78% yield) of a fluffy white solid. This material is vacuum-dried overnight at 50°C/5 Torr to give 14.46 g (0.2% w/w loss on drying) of a fluffy white solid.

Formulation

The salts of the present invention are preferably formulated in a unit dosage form prior to administration to the recipient patient. The term "patient" includes human and non-human animals such as companion animals (dogs and cats and the like). Therefore, yet another embodiment of the present invention is a pharmaceutical composition comprising a salt of the present invention and a pharmaceutical carrier. The term "pharmaceutical" when used herein as an adjective means substantially non-deleterious to the recipient patient.

The present pharmaceutical formulations are prepared by known procedures using well-known and readily available ingredients. In making the formulations of the present invention, the active ingredient (a crystalline salt of the present invention) will usually be mixed with a carrier, or diluted by a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet, paper or other container. When the carrier serves as a diluent, it may be a solid, semisolid or liquid material that acts as a vehicle, excipient or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosol (as a solid or in a liquid medium), soft and hard gelatin capsules, suppositories, sterile injectable solutions and sterile packaged powders.

Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water syrup, methyl cellulose, methyl and propylhydroxybenzoates, talc, magnesium stearate and mineral oil. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions of the invention may be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the recipient patient.

Formulation Example 1: 5mg Capsule

Ingredient	Quantity (mg/capsule)
Hemi-fumarate F-I	5.6
Mannitol	177.2-201.2
Microcrystalline Cellulose	177.2-201.2
Povidone	8.0-16.0
Sodium Lauryl Sulfate	1.0-3.0
Magnesium Stearate	1.0-3.0

Formulation Example 2: 25mg Capsule

Ingredient	Quantity (mg/capsule)
Hemi-fumarate F-I	28.2
Mannitol	170.4-195.0
Microcrystalline Cellulose	170.4-195.0
Povidone	8.2-16.4
Sodium Lauryl Sulfate	1.0-3.1
Magnesium Stearate	1.0-3.1

The capsules above are manufactured by an aqueous granulation process. The mannitol, microcrystalline cellulose, and active ingredient are added to the granulator and dry blended for a suitable period of time to uniformly distribute the powders. A previously prepared granulation solution consisting of purified water, povidone, and sodium lauryl sulfate is sprayed at a uniform rate onto the powders while mixing. When a suitable granulation endpoint is reached, the granulator is stopped and the granulation is unloaded. The granulation is then wet sieved, through a suitable screen to disrupt large agglomerates, spread on paper lined trays, and dried in a convection oven until the moisture is reduced to a suitable level. The size of the granulation is adjusted to a range consistent with automated capsule filling equipment requirements by passing through a co-mill or other suitable apparatus. These powders are collected and transferred to a mixing apparatus with a specified quantity of magnesium stearate. The entire powder mixture is blended for a suitable length of time to uniformly distribute the lubricant. The finished powders are then filled into hard gelatin capsules on a suitable piece of automated capsule filling equipment. Following the filling operation, the finished capsules are visually inspected for external defects. To improve the pharmaceutical elegance of the finished product, the capsules may be physically de-dusted and polished by either manual or automated processes.

Demonstration of Function

The genes encoding the human β_1-adrenergic receptor (Frielle *et al.*, *Proc. Natl. Acad. Sci.*, 84:7920-7924, 1987), the human β_2-adrenergic receptor (Kobika *et al.*, *Proc. Natl. Acad. Sci.*, 84:46-50, 1987, Emorine *et al.*, *Proc. Natl. Acad. Sci.*, 84:6995-6999,

1987) and the human β3 adrenergic receptor (Granneman *et al.*, *Molecular Pharmacology*, 44(2):264-70, 1993) are individually subcloned into a phd expression vector (Grinnell *et al.*, *Bio/Technology*, 5:1189-1192, 1987) and transfected into the DXB-11 Chinese hamster ovary (CHO) cell line by calcium phosphate precipitation methodology. The stably transfected cells are grown to 95% confluency in 95% Dulbecco's modified Eagles Medium (DMEM), 5% fetal bovine serum and 0.01% proline. Media is removed and the cells are washed with phosphate buffered (pH 7.4) saline (without magnesium and calcium). Cells are then lifted using an enzyme free cell dissociation solution (Specialty Media, Lavallette, New Jersey) and pelleted by centrifugation.

Cells from each of the above cell lines are resuspended and added (20,000/well) to a 96-well plate. Cells are incubated at 37°C with representative compounds of the invention for 20 minutes in buffer (Hank's balanced salt solution, 10 mM HEPES, 0.1% BSA, 1 mM L-ascorbic acid, 0.2% dimethyl sulfoxide, 1 mM 3-isobutyl-1-methylxanthine, pH 7.4). After halting the incubation with quench buffer (50 mM Na Acetate, 0.25% Triton X-100, pH 5.8), the c-AMP level is quantified by scintillation proximity assay (SPA) using a modification of the commercially available c-AMP kit (Amersham, Arlington Heights, IL) with rabbit anti-cAMP antibody (ICN Biomedicals, Aurora, Ohio) for the kit.

Sigmoidal dose response curves, from the whole cell receptor coupled c-AMP assay are fit to a four parameter logistic equation using non linear regression: $y=(a-d)/(1+(Dose/c)^b)+d$ where a and d are responses at zero and maximal dose, b is the slope factor and c is the EC_{50} as previously described (DeLean et al., *Am. J. Physiol.*, 235, E97-E102, 1978). EC_{50} is assessed as the concentration producing 50% of the maximum response to each agonist.

Isoproterenol is accepted in the art as a non-selective β_3 agonist and is widely used as a comparator in evaluating the activity of compounds. See *Trends in Pharm. Sci.*, 15:3, 1994. The % intrinsic activity (E_{max}) of representative salts of the present invention were assessed relative to isoproterenol by the compound's maximal response divided by the isoproterenol maximal response times 100. E_{max} and Standared Error Mean (SEM) data generated for these salts is presented below in Table 6. The E_{max} values represent the average of three runs.

Table 6

Salt	Emax (SEM)
Hemi-fumarate hemi-hydrate	85.8 (6.3)
Benzoate	83.6 (3.74)
(R)-Mandalate	86.4 (0.93)
Salicylate	97.6 (5.78)

In vitro Rat Atrial Tachycardia

Male Sprague-Dawley rats (250-400 grams) (Harlan Sprague-Dawley, Indianapolis, Indiana USA) are killed by cervical dislocation. Hearts are removed and the left and right atria are dissected and mounted with thread in tissue baths containing 10 ml of modified Krebs' solution of the following composition (mM concentrations): NaCl 118.2, KCl 4.6, $CaCl_2 \cdot 2H_2O$ 1.6, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 24.8. An initial resting force of 1 gram is applied to the atria (Cohen et al, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 320:145-152, 1982). Tissues are allowed to equilibrate approximately 30 minutes with vigorous oxygenation before exposure to drugs. Concentrations of SAM II dissolved in polyethylene glycol 300 and saline, are added cumulatively every 4-5 minutes. SAM II addition is continued until no further increase in atrial rate occurred or until a concentration of 10^{-4}M is reached. The increase in beats per minute (bpm) is measured with Sensotec transducers (model MBL-5514-02) for each concentration of SAM II and recorded by a Biopac Data Acquisition System. Spontaneously beating rat atria received either vehicle or SAM II.

SAM II did not increase heart rate (n=3; 1.8 ± 0.07% increase above starting heart rate) relative to vehicle (n=8; 3.5 ± 0.6% increase in heart rate above starting heart rate). No significant difference is observed in the initial heart rates in the two groups studied. These results are expressed as mean ± the standard error of the mean where n represents the number of isolated atria examined. These data are expressed as a percent of the basal increase in heart rate and as a change in heart rate in beats per minute (bpm) from basal heart rates.

Utilities

As agonists of the β_3 receptor, the salts of the present invention are useful in treating conditions in human and non-human animals in which the β_3 receptor has been demonstrated to play a role. The terms "treating" and "treat", as used herein, include their generally accepted meanings, *i.e.*, alleviating, ameliorating, managing, preventing, prohibiting, restraining, slowing, stopping, or reversing the progression or severity of a pathological condition, or sequela thereof, described herein. The term "preventing" refers to reducing the likelihood that the recipient patient of a salt of the present invention will incur or develop any of the pathological conditions, or sequela thereof, described herein.

The diseases, disorders or conditions for which compounds of the present invention are useful in treating include, but are not limited to, (1) diabetes mellitus, (2) hyperglycemia, (3) obesity, (4) hyperlipidemia, (5) hypertriglyceridemia, (6) hypercholesterolemia, (7) atherosclerosis of coronary, cerebrovascular and peripheral arteries, (8) hypertension, (9) disorders of the gall bladder including acute and chronic cholecystitis, (10) depression, (11) elevated intra-ocular pressure and glaucoma, (12) non-specific diarrhea dumping syndrome, (13) hepatic steatosis [fatty degeneration of the liver], and obesity dependent diseases/disorders such as: (14) gastrointestinal disorders including peptid ulcer, esophagitis, gastritis and duodenitis, (including that induced by H. pylori), intestinal ulcerations (including inflammatory bowel disease, ulcerative colitis, Crohn's disease and proctitis) and gastrointestinal ulcerations, (15) irritable bowel syndrome and other disorders needing decreased gut motility, (16) diabetic retinopathy, (17) neuropathic bladder dysfunction, (18) osteoarthritis, (19) restrictive lung disease, (20) obstructive sleep apnea, (21) congestive heart failure, (22) venous stasis and skin disorders related to venous stasis, (23) decreased libido (in both males and females), and (24) acute and chronic cystitis. The term "obesity dependent" means that the symptoms of said diseases will be ameliorated via the present salt's effect on the patient's weight.

Human patients in need of obesity treatment are typically those with a body mass index (BMI) >27 or those with a BMI ≥25 when co-morbidities, e.g., hypertension, sleep apnea and/or osteoarthritis, are present. A patient population at particular need of treatment are those with a BMI >30 or >27 with co-morbities.

Human patients in need of hypertension treatment are frequently overweight individuals, i.e., those with a BMI ≥25, but may also be of normal body weight (*i.e.*, BMI <25).

Human patients in need of type 2 diabetes treatment are typically individuals with a BMI <25, *i.e.*, individuals that are not overweight.

Dose

As used herein, the term "effective amount" means an amount of a salt of the present invention that is capable of treating the conditions described herein or that is capable of agonizing the β_3 receptor.

The specific dose or amount administered is determined by the particular circumstances surrounding each situation. These circumstances include, the route of administration, the prior medical history of the recipient, the pathological condition or symptom being treated, the severity of the condition/symptom being treated, and the age and sex of the recipient patient. However, it will be understood that the therapeutic dosage administered will be determined by the physician in the light of the relevant circumstances.

Generally, an effective minimum daily dose of a salt of the present invention will exceed about 5 mg. Typically, an effective maximum daily dose will not exceed about 350 mg. The exact dose may be determined, in accordance with the standard practice in the medical arts of "dose titrating" the recipient; that is, initially administering a low dose of the compound, and gradually increasing the does until the desired therapeutic effect is observed.

Route of Administration

The crystalline salts of the present invention can be administered by a variety of routes including the oral, rectal, transdermal, subcutaneous, topical, intravenous, intramuscular or intranasal routes. A preferred route of administration is the oral route.

Combination Therapy

The crystalline salts of the present invention may be used in combination with other drugs that are used in the treatment of the diseases or conditions for which the present salts are useful, e.g., treatment of obesity and/or type 2 diabetes. Such other drug(s) may be administered, by a route and in an amount commonly used therefore, contemporaneously or sequentially with a salt of the present invention. When a salt of the present invention is used contemporaneously with one or more other drugs, a pharmaceutical unit dosage form containing such other drugs in addition to the present salt is preferred. Accordingly, the pharmaceutical compositions of the present invention include those that also contain one or more other active ingredients, in addition to a salt of the present invention.

A preferred combination therapy for the treatment of obesity is the use of a salt of the present invention in combination with sibutramine (or active metabolites of sibutramine, *e.g.*, desmethyl sibutramine and di-desmethyl sibutramine), preferably with sibutramine hydrochloride mono-hydrate.

We claim:

1. A crystalline pharmaceutical acid addition salt of 2-(4-{2-[2-hydroxy-3-(2-thiophen-2-yl-phenoxy)-propylamino]-2-methyl-propyl}-phenoxy)-nicotinonitrile.

2. The salt of claim 1 which is the non-solvated hemi-fumarate.

3. The hemi-fumarate of claim 2 having an X-ray diffraction pattern which comprises the following peaks: 11.4 ± 0.1, 17.6 ± 0.1, 17.9 ± 0.1 and 20.3 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

4. The hemi-fumarate of Claim 3 wherein said X-ray diffraction pattern further comprises the following peaks: 18.6 ± 0.1, 18.8 ± 0.1, 19.4 ± 0.1 and 27.1 ± 0.1° in 2θ.

5. The salt of claim 1 which is the hemi-fumarate hemi-hydrate.

6. The hemi-hydrate of claim 5 having an X-ray diffraction pattern which comprises the following peaks: 11.4 ± 0.1, 12.7 ± 0.1, 18.6 ± 0.1 and 21.3 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

7. The hemi-hydrate of claim 6 wherein said X-ray diffraction pattern further comprises the following peaks: 8.4 ± 0.1, 9.9 ± 0.1, 15.2 ± 0.1 and 23.8 ± 0.1° in 2θ.

8. The hemi-hydrate of claim 6 or 7 wherein said X-ray diffraction pattern further comprises the following peaks: 4.2 ± 0.1 and 7.8 ± 0.1° in 2θ.

9. The salt of claim 1 which is the non-solvated benzoate.

10. The benzoate of claim 9 having an X-ray diffraction pattern which comprises the following peaks: 8.6 ± 0.1,14.9 ± 0.1, 19.3 ± 0.1 and 22.2 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

11. The benzoate of Claim 10 wherein said X-ray diffraction pattern further comprises the following peaks: 17.2 ± 0.1,17.6 ± 0.1, 18.7 ± 0.1 and 20.4 ± 0.1° in 2θ.

12. The salt of claim 1 which is the non-solvated (R)-mandalate.

13. The mandalate of claim 12 having an X-ray diffraction pattern which comprises the following peaks: 4.7 ± 0.1, 13.2 ± 0.1, 21.1 ± 0.1 and 21.8 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

14. The mandalate of Claim 13 wherein said X-ray diffraction pattern further comprises the following peaks: 16.9 ± 0.1, 18.2 ± 0.1, 18.6 ± 0.1 and 20.0 ± 0.1° in 2θ.

15. The salt of claim 1 which is the non-solvated salicylate.

16. The salicylate of claim 15 having an X-ray diffraction pattern which comprises the following peaks: 14.6 ± 0.1, 16.9 ± 0.1, 18.0 ± 0.1 and 22.6 ± 0.1° in 2θ; when the pattern is obtained from a copper radiation source (λ = 1.54056).

17. The salicylate of Claim 16 wherein said X-ray diffraction pattern further comprises the following peaks: 6.9 ± 0.1, 8.2 ± 0.1, 8.8 ± 0.1 and 19.0 ± 0.1° in 2θ.

18. A pharmaceutical composition comprising a salt of any one of claims 1-17 and a pharmaceutical carrier.

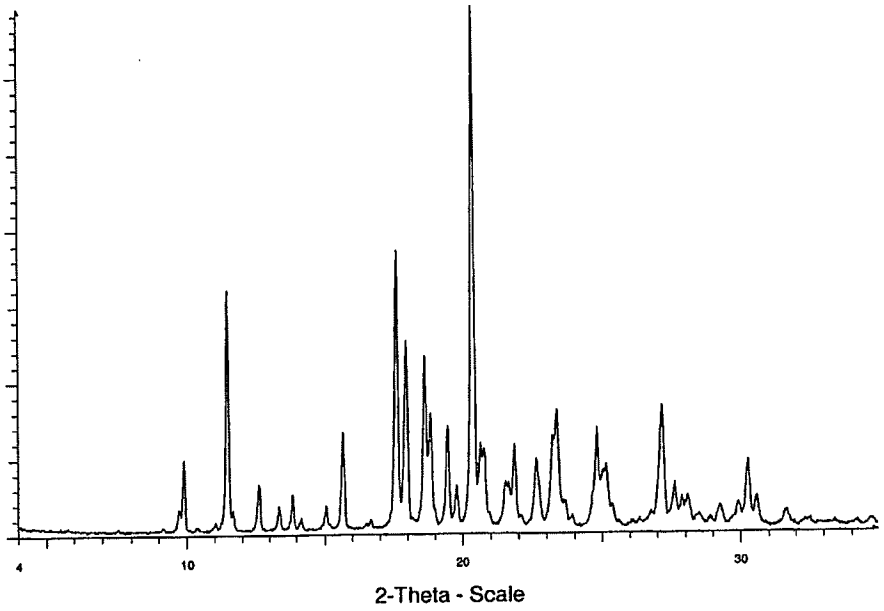
19. A method of agonizing the β_3 receptor comprising administering to a patient in need thereof an effective amount of a salt of any one of claims 1-17.

20. A method of treating obesity comprising administering to a patient in need thereof an effective amount of a salt of any one of claims 1-17.

21. A method of treating type 2 diabetes comprising administering to a patient in need thereof an effective amount of a salt of any one of claims 1-17.

22. A method of treating hypertension comprising administering to a patient in need thereof an effective amount of a salt of any one of claims 1-17.

23. The salt of any one of claims 1-17 for use in treating type 2 diabetes, obesity or hypertension or for use in agonizing the β_3 receptor.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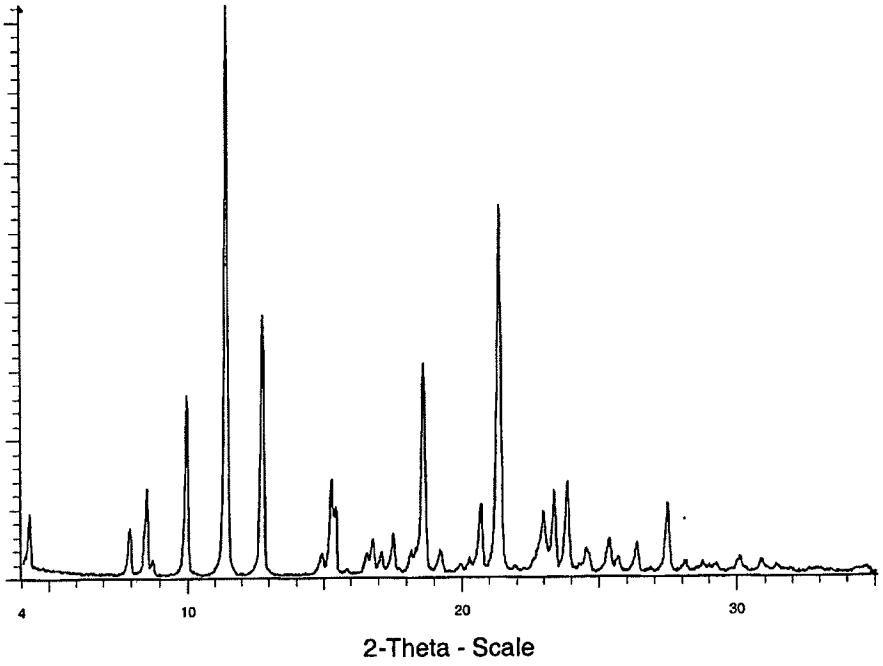
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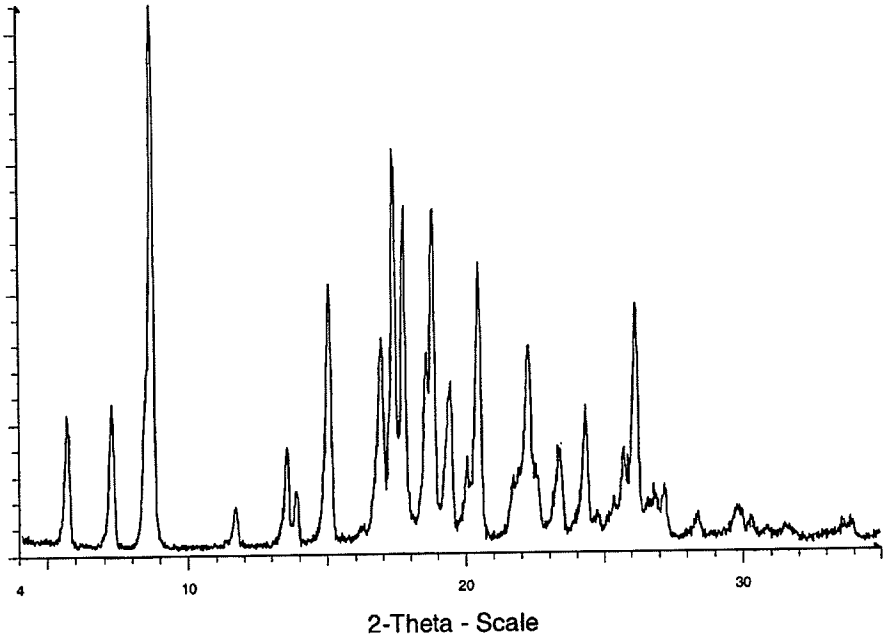
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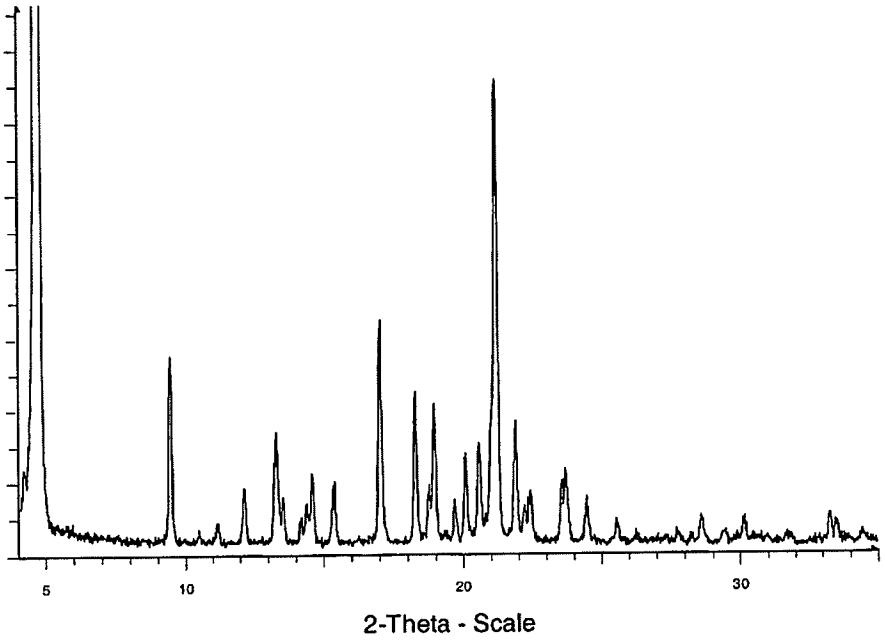
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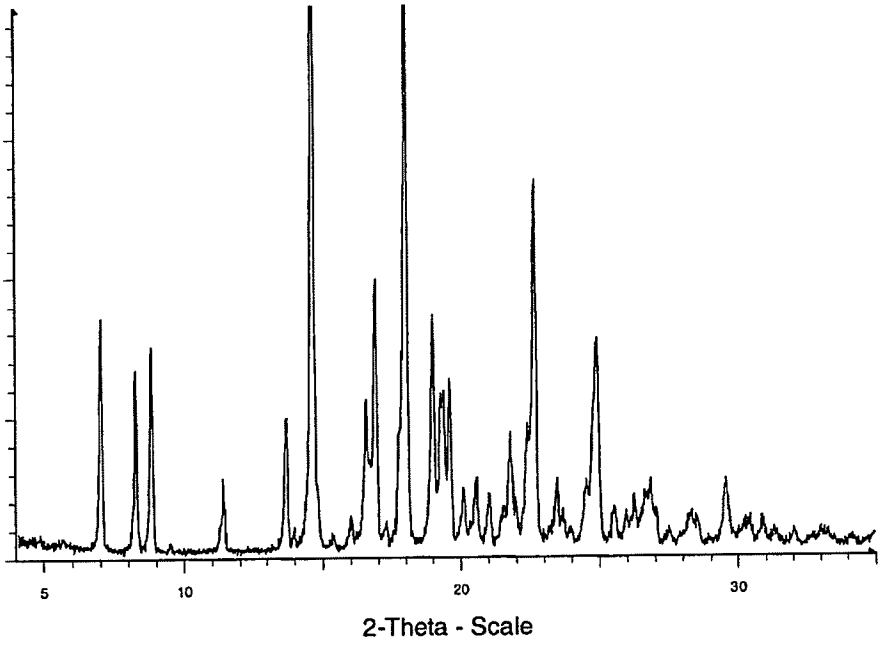
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INTERNATIONAL SEARCH REPORT

tional Application No
PCT/US 02/11896

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D409/12 A61K31/44 //(C07D409/12,333:00,213:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 236 624 A (BEECHAM GROUP PLC) 16 September 1987 (1987-09-16) the whole document	1-23
A	US 5 977 154 A (RITO CHRISTOPHER JOHN ET AL) 2 November 1999 (1999-11-02) the whole document	1-23
A	EP 0 678 511 A (SANKYO CO) 25 October 1995 (1995-10-25) the whole document	1-23

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 August 2002	27/08/2002

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INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 02/11896

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 19-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0236624 A	16-09-1987	DE 3688885 D1	16-09-1993
		EP 0236624 A2	16-09-1987
		JP 62175458 A	01-08-1987
		US 5153210 A	06-10-1992
US 5977154 A	02-11-1999	US 6093735 A	25-07-2000
		US 6265581 B1	24-07-2001
		US 5808080 A	15-09-1998
		US 6075040 A	13-06-2000
		US 5939443 A	17-08-1999
		US 5786356 A	28-07-1998
		US 6060492 A	09-05-2000
		US 5840738 A	24-11-1998
		AU 715175 B2	20-01-2000
		AU 7077896 A	09-04-1997
		BR 9610852 A	13-07-1999
		CA 2232434 A1	27-03-1997
		CN 1202107 A	16-12-1998
		CZ 9800820 A3	12-08-1998
		EP 0764640 A1	26-03-1997
		HU 9802814 A2	28-10-1999
		IL 134420 A	13-09-2001
		JP 11512701 T	02-11-1999
		NO 981203 A	06-05-1998
		NZ 318718 A	28-10-1999
		PL 327408 A1	07-12-1998
		TR 9800518 T1	22-06-1998
		WO 9710825 A1	27-03-1997
EP 0678511 A	25-10-1995	AU 682435 B2	02-10-1997
		AU 1501695 A	05-10-1995
		CA 2145257 A1	24-09-1995
		CN 1132750 A ,B	09-10-1996
		CZ 9500739 A3	13-12-1995
		EP 0678511 A2	25-10-1995
		FI 951370 A	24-09-1995
		HU 71231 A2	28-11-1995
		IL 113084 A	26-01-1999
		JP 7330710 A	19-12-1995
		NO 951095 A	25-09-1995
		NZ 270793 A	27-02-1996
		US 5578620 A	26-11-1996
		US 5688823 A	18-11-1997
		ZA 9502383 A	14-12-1995

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: USE OF A β_2 ADRENERGIC RECEPTOR AGONIST IN THE TREATMENT OF CARDIOVASCULAR DISEASE

(57) Abstract

The present invention provides a method of using a β_2 adrenergic receptor agonist that selectively activates Gs proteins in the treatment of cardiovascular disease. More particularly, the present invention provides a method of using fenoterol or an acid addition salt thereof to activate β_2AR Gs proteins selectively in the treatment of acute heart failure, chronic heart failure, aging heart and the like.

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USE OF A β_2 ADRENERGIC RECEPTOR AGONIST IN THE TREATMENT OF CARDIOVASCULAR DISEASE

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method of using a β_2-adrenergic receptor agonist that selectively activates G_s proteins in the treatment of cardiovascular disease. In particular, the present invention relates to a method of using fenoterol to activate selectively G_s proteins in the treatment of acute heart failure, chronic heart failure and aging heart.

BACKGROUND OF THE INVENTION

Heart failure is a disease of left ventricular dysfunction accompanied by severely impaired β-adrenergic receptor-mediated contractile response (Bristow et al., N. Engl. J. Med. 307: 205 (1982); Bristow et al., Circ. Res. 59: 297 (1986); Brodde, Pharmacol. Rev. 43: 203 (1991); Marzo et al., Circ. Res. 69: 1546 (1991); Feldman, Circulation 87 (Suppl.): IV27 (1993); Kiuchi et al., J. Clin. Invest. 91: 907 (19930: Yamamoto et al., J. Mol. Cell. Cardiol. 26: 617 (1994); and Brodde et al., J. Cardiovasc. Pharmacol. 31: 585 (1998)) associated with a selective down-regulation of the β_1 adrenergic receptor (higher β_2/β_1 ratio) (Bristow et al. (1982), *supra*; Bristow et al. (1986), *supra*; Brodde (1991), *supra*; Marzo et al. (1991), *supra*; and Feldman (1993), *supra*) and increases in inhibitory G protein (G_i) mRNA level, protein abundance and activity (Feldman et al., J. Clin. Invest. 82: 189 (1988); Neumann et al., Lancet 2: 936 (1988); Eschenhagen et al., Circ. Res. 70: 688 (1992); Bohm et al., Hypertension 22: 715 (1993); Spinale et al., Cardiovasc. Res. 28: 1243 (1994); Ping et al., Am. J. Physiol. 267: H2079 (1994); Steinberg et al., Circulation 91: 2824 (1995); and Shi et al., Am. J. Physiol. 269: H1073 (1995)). Heart failure can be acute or chronic. Chronic heart failure has been the number one killer in the United States, causing more than 950,000 lives every year in the United States alone. Consequently, billions of dollars are spent every year in the treatment of chronic heart failure – at a cost to families and the U.S. government. Therefore, the treatment of this disease has become extremely important for health care and economic reasons. Also important for the same reasons is the treatment of the aging heart, which, given the ever increasing elderly population, is becoming increasingly costly.

In the heart, β-adrenergic receptor (βAR) stimulation provides the primary regulatory mechanism on cardiac function. There are at least two βAR subtypes, namely β_1AR and β_2AR, that exist in the myocardium, although β_1AR predominates.

While β_1AR couples to stimulatory G proteins (G_s), β_2AR elicits bifurcated signaling pathways mediated by G_s and G_i, resulting in functionally opposing effects on cardiac function. In failing and aged hearts, the overall response to βAR stimulation is markedly diminished due to a down-regulation of β_1AR and an up-regulation of G_i proteins.

βAR agonists are used in the treatment of heart failure. However, most of the βAR agonists used target β_1-adrenergic receptors (β_1AR) or β_1AR and β_2AR and those that target β_2AR do not selectively activate G_s or G_i proteins. This, in part, has led to the prevalent view that βAR stimulation in failing heart is deleterious – not beneficial.

The present invention is predicated on the surprising and unexpected discovery that a β_2AR agonist can selectively activate G_s proteins. The present invention is further predicated on the surprising and unexpected discovery that selective activation of G_s proteins by a β_2AR agonist can revive βAR contractile support in failing hearts.

The present invention is further predicated on the surprising and unexpected discovery that fenoterol (U.S. Patent No. 3,341,593), otherwise known as 5-[1-hydroxy-2-[[2-(4-hydroxyphenyl)-1-methylethyl]amino]ethyl]-1,3-benzenediol or 3,5-dihydroxy-α-[[(p-hydroxy-α-methylphenethyl)amino]methyl]benzyl alcohol or 1-(3,5-dihydroxyphenyl)-1-hydroxy-2-[(4-hydroxy-phenyl)isopropylamino]ethane or 1-(p-hydroxyphenyl)-2-[[β-hydroxy-β-(3',5'-dihydroxyphenyl)]ethyl]aminopropane, activates β_2AR G_s proteins without activating β_2AR G_i proteins and consequently revives βAR contractile support in failing hearts. While fenoterol was known to be a β_2AR agonist, it was not known to have selective β_2AR G_s activation activity and to be useful in the treatment of acute heart failure, chronic heart failure and aging heart; rather, it was known for its bronchodilatory and tocolytic effects (Merck Index) and its use in the treatment of acute circulatory arrest, shock, and acute respiratory insufficiency in status asthmaticus and chronic lung diseases (Schuster et al., Arzneimittel-forsch. 19: 1905-1914 (1969)). Not only does fenoterol revive βAR contractile support in failing hearts, but it does so effectively and so selectively for βAR G_s proteins that low doses can be used, thereby minimizing the adverse side effects realized by activation of G_i proteins and high doses of fenoterol.

In view of the above, it is an object of the present invention to provide ligands, i.e., agonists and antagonists, and methods for the selective activation and inactivation of a subset of signaling pathways coupled to any given receptor of any cell or tissue type. It is another object of the present invention to provide ligands, i.e., agonists and antagonists, and methods for the selective activation and inactivation of a subset of

signaling pathways, such as pathways involving G proteins, in particular G_s and G_i proteins, coupled to a cardiovascular receptor, such as β_2AR, for the treatment of cardiovascular disease. It is yet another object of the present invention to provide a method of using a β_2AR agonist to activate selectively G_s proteins in the treatment of cardiovascular disease. It is still yet another object of the present invention to provide a method of using fenoterol (or a pharmaceutically acceptable salt thereof) to activate selectively G_s proteins in the treatment of acute heart failure, chronic heart failure, aging heart and the like. These and other objects and advantages, as well as additional inventive features, of the present invention will become apparent to one of ordinary skill in the art upon reading the detailed description of the present invention provided herein.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of using a β_2 adrenergic receptor agonist that selectively activates G_s proteins in the treatment of cardiovascular disease. The method comprises administering to a mammal, such as a human, in need thereof, a treatment effective amount of the β_2 adrenergic receptor agonist so as to treat cardiovascular disease. Preferably, the β_2 adrenergic receptor agonist is fenoterol or an acid addition salt thereof and the cardiovascular disease is acute heart failure, chronic heart failure, aging heart or the like.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of using a β_2 adrenergic receptor agonist that selectively activates G_s proteins in the treatment of cardiovascular disease. The method comprises administering to a mammal in need thereof a treatment effective amount of the β_2 adrenergic receptor agonist so as to treat cardiovascular disease.

In a preferred embodiment, the present invention provides a method of using the β_2 adrenergic receptor agonist fenoterol or an acid addition salt thereof in the treatment of acute heart failure, chronic heart failure, aging heart and the like. The method comprises administering to a mammal in need thereof a treatment effective amount of fenoterol or an acid addition salt thereof so as to treat acute heart failure, chronic heart failure, aging heart and the like.

Any β_2 adrenergic receptor agonist that selectively activates G_s proteins can be used in the method of the present invention as long as it is safe and efficacious. Preferably, the agonist is fenoterol or an acid addition salt thereof. Whether or not a particular β_2 adrenergic receptor agonist can selectively activate G_s proteins can be

determined using the methods set forth in the examples. In this regard, the β_2AR agonists zinterol, procaterol and salbutamol, unlike fenoterol, concurrently activated G_s and G_i proteins.

Fenoterol is commercially available. However, the synthesis of fenoterol and its acid addition salts is described in U.S. Patent No. 3,341,593.

Desirably, the β_2 adrenergic receptor agonist that selectively activates G_s proteins is administered as soon as possible after cardiovascular disease has been diagnosed. One skilled in the art will appreciate that suitable methods of administering a β_2 adrenergic receptor agonist, which selectively activates G_s proteins and is useful in the method of the present invention, are available. Although more than one route can be used to administer the agonist, a particular route can provide a more immediate and more effective reaction than another route. Accordingly, the described methods are merely exemplary and are in no way limiting.

The dose administered to an animal, particularly a human, in accordance with the present invention should be sufficient to effect the desired response in the animal over a reasonable time frame. One skilled in the art will recognize that dosage will depend upon a variety of factors, including the strength of the particular agonist employed, the age, species, overall condition, and body weight of the animal, as well as the degree of cells or tissues affected, e.g., the degree of cardiac myocytes affected by cardiovascular disease, such as acute heart failure, chronic heart failure or aging heart. The size of the dose also will be determined by the route, timing and frequency of administration as well as the existence, nature, and extent of any adverse side-effects that might accompany the administration of a particular agonist. It will be appreciated by one of ordinary skill in the art that prolonged treatment involving multiple administrations can be required.

Suitable doses and dosage regimens can be determined by conventional range-finding techniques known to those of ordinary skill in the art. Generally, treatment is initiated with smaller dosages, which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect, e.g., prophylactic or therapeutic treatment, under the circumstances is reached. The present inventive method will typically involve the administration of about 0.001 to about 1000 mg, preferably about 0.01 to about 100 mg, of an agonist per kg treated weight. See, for example, Goldenthal, Toxicol. Appl. Pharmacol. 18: 185-207 (1971), and Kojima et al., Arzneimittel-forsch. 30: 959- 964 (1980).

Compositions for use in the present inventive method preferably comprise a pharmaceutically acceptable carrier and an amount of an agonist sufficient to treat cardiovascular disease. The carrier can be any of those conventionally used and is

limited only by chemico-physical considerations, such as solubility and lack of reactivity with the compound, and by the route of administration. It will be appreciated by one of skill in the art that, in addition to the following described pharmaceutical composition, the agonist can be formulated as inclusion complexes, such as cyclodextrin inclusion complexes, or liposomes.

The agonist can be formulated as a pharmaceutically acceptable acid addition salt. Examples of pharmaceutically acceptable acid addition salts for use in the pharmaceutical composition include those derived from mineral acids, such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric and sulfuric acids, and organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, and arylsulphonic, for example p-toluenesulphonic, acids.

The pharmaceutically acceptable excipients described herein, for example, vehicles, adjuvants, carriers or diluents, are well-known to those who are skilled in the art and are readily available to the public. It is preferred that the pharmaceutically acceptable carrier be one that is chemically inert to the agonist and has no detrimental side effects or toxicity under the conditions of use.

The choice of excipient will be determined in part by the particular agonist, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical composition of the present invention. The following formulations for oral, aerosol, parenteral, subcutaneous, intravenous, intramuscular, intracardiac and interperitoneal administration are merely exemplary and are in no way limiting.

Injectable formulations are among those formulations that are preferred in accordance with the present inventive method. The requirements for effective pharmaceutical carriers for injectable compositions are well known to those of ordinary skill in the art (See Pharmaceutics and Pharmacy Practice, J.B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250, (1982), and ASHP Handbook on Injectable Drugs, Toissel, 4th ed., pages 622-630 (1986)). It is preferred that such injectable compositions be administered intravenously, intramuscularly, subcutaneously or intracardially.

Topical formulations are well-known to those of skill in the art and are suitable in the context of the present invention for application to skin.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or orange juice; (b) capsules, sachets, tablets, lozenges, and troches, each containing a predetermined amount of the active ingredient, as solids or granules; (c) powders; (d) suspensions in an appropriate liquid; and (e) suitable

emulsions. Liquid formulations may include diluents, such as water and alcohols, for example, ethanol, benzyl alcohol, and the polyethylene alcohols, either with or without the addition of a pharmaceutically acceptable surfactant, suspending agent, or emulsifying agent. Capsule forms can be of the ordinary hard- or soft-shelled gelatin type containing, for example, surfactants, lubricants, and inert fillers, such as lactose, sucrose, calcium phosphate, and corn starch. Tablet forms can include one or more of lactose, sucrose, mannitol, corn starch, potato starch, alginic acid, microcrystalline cellulose, acacia, gelatin, guar gum, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, calcium stearate, zinc stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, disintegrating agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, gels, and the like containing, in addition to the active ingredient, such excipients as are known in the art.

The agonist used in the present inventive method, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They also can be formulated as pharmaceuticals for non-pressured preparations, such as in a nebulizer or an atomizer. Such spray formulations may be used to spray mucosa. Aerosol formulations are not preferred for administration of fenoterol in the treatment of acute heart failure, chronic heart failure, aging heart and the like.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The agonist can be administered in a physiologically acceptable diluent in a pharmaceutical carrier, such as a sterile liquid or mixture of liquids, including water, saline, aqueous dextrose and related sugar solutions, an alcohol, such as ethanol, isopropanol, or hexadecyl alcohol, glycols, such as propylene glycol or polyethylene glycol, dimethylsulfoxide, glycerol ketals, such as 2,2-dimethyl-1,3-dioxolane-4-methanol, ethers, such as poly(ethyleneglycol) 400, an oil, a fatty acid, a fatty acid ester or glyceride, or an acetylated fatty acid glyceride with or without the addition of

a pharmaceutically acceptable surfactant, such as a soap or a detergent, suspending agent, such as pectin, carbomers, methylcellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agents and other pharmaceutical adjuvants.

Oils, which can be used in parenteral formulations include petroleum, animal, vegetable, or synthetic oils. Specific examples of oils include peanut, soybean, sesame, cottonseed, corn, olive, petrolatum, and mineral.

Suitable fatty acids for use in parenteral formulations include oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters.

Suitable soaps for use in parenteral formulations include fatty alkali metals, ammonium, and triethanolamine salts, and suitable detergents include (a) cationic detergents such as, for example, dimethyl dialkyl ammonium halides, and alkyl pyridinium halides, (b) anionic detergents such as, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates, (c) nonionic detergents such as, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylenepolypropylene copolymers, (d) amphoteric detergents such as, for example, alkyl-β-aminopropionates, and 2-alkyl-imidazoline quaternary ammonium salts, and (e) mixtures thereof.

The parenteral formulations will typically contain from about 0.5 to about 25% by weight of the active ingredient in solution. Preservatives and buffers may be used. In order to minimize or eliminate irritation at the site of injection, such compositions may contain one or more nonionic surfactants having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulations will typically range from about 5 to about 15% by weight. Suitable surfactants include polyethylene sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol. The parenteral formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

In view of the above, the β_2 adrenergic receptor agonist that selectively activates G_s proteins can be administered alone or in combination with one or more other active ingredients as are known in the art.

Furthermore, such β_2 adrenergic receptor agonists that selectively activate G_s proteins are also useful in the research and development of prophylactic and therapeutic treatment of cardiovascular disease, such as acute heart failure, chronic heart failure and aging.

EXAMPLES

The following examples serve to illustrate further the present invention and are not intended to limit the scope of the present invention.

Example 1

This example demonstrates that fenoterol selectively activates the β_2AR-coupled G_s signaling pathway in isolated cardiac myocytes *in vitro*.

Rat cardiac myocytes, which were treated with pertussis toxin (PTX) or left untreated, were exposed to the β_2AR agonist fenoterol and contractile response was measured. The measurement of contractile response in the presence of PTX is indicative of G_s protein activation, whereas the measurement of contractile response in the absence of PTX is indicative of combined G_s and G_i activation. Fenoterol induced a maximal contractile response of approximately 300% of control in the absence of PTX. The presence of PTX over a wide range of concentrations (10^{-8} M to 10^{-4}M) did not affect the contractile response induced by fenoterol. These results indicate that fenoterol selectively activates the β_2AR-coupled G_s signaling pathway. Similar selective activation results were obtained in mouse cardiac myocytes. Preliminary data also show similar contractile response to fenoterol (10^{-9}M to 10^{-6}M) in isolated, failing human myocytes.

Example 2

This example demonstrates fenoterol selectively activates the β_2AR-coupled G_s signaling pathway in cardiac myocytes, which are essentially β_1AR-free, *in vitro*.

A transgenic murine (TG4) model in which the human β_2AR is overexpressed by approximately 200 fold in a cardiac-specfic manner (Milano et al., Science 264: 582 (1994)) was used. β_1AR is essentially nonfunctional in the hearts of TG4 transgenic mice *in vivo* (Du et al., Am. J. Physiol. 271: H630 (1996)), in isolated atria (Bond et al., Nature 374: 272 (1995); and Milano et al. (1994), *supra*) and in PTX-treated and untreated cardiac cells. Accordingly, any cellular response to a βAR agonist is mediated almost exclusively through the nearly homogeneous population of β_2AR.

Myocytes were isolated from TG4 and wild-type littermates using a slightly modified enzymatic technique of Korzick et al., Am. J. Physiol. 41: H590 (1997). Aliquots of cells were incubated with PTX (1.5 μg/ml at 37 °C for at least 3 hr) to abrogate G_i protein function via ribosylation, as previously described (Xiao et al., ibid. 47: 322 (1995); Xiao et al., J. Clin Ivest. 101: 1273 (1998); and Zhou et al., Am. J. Physiol. 273: H1611 (1997)). After PTX treatment, cells were kept at room temperature for the rest of the experimental day (approximately 6-8 hr). PTX-treated cells were compared with myocytes from the same heart that had been kept at 37 °C in the absence of PTX for an equal time. Cells were then perfused with Hepes buffer solution consisting of (in mM) 1.0 $CaCl_2$, 137 NaCl, 5 KCl, 15 dextrose, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, and 20 Hepes, pH 7.4, and were electrically stimulated at 0.5 Hz at 23 °C. Cell length was monitored from the brightfield image of the cell by an optical edge-tracking method using a photodiode array (Reticon Model 1024 SAQ) with a 3 ms time resolution. Cell contraction was indexed by the percent reduction of cell length following electrical stimulation.

In PTX-treated and untreated TG4 myocytes, fenoterol (10^{-9} M to 10^{-4} M) induced a dose-dependent increase in cell contraction. In fact, PTX did not potentiate the effect of fenoterol on the contractile response, indicating that an uncoupling of fenoterol stimulated β_2AR from G_i proteins still occurs in cardiac myocytes that overexpress β_2AR.

Example 3

This example demonstrates that fenoterol selectively activates the β_2AR-coupled G_s signaling pathway in isolated cardiac myocytes *in vitro*.

Rat cardiac myocytes, which were treated with PTX or left untreated, were exposed to fenoterol and cAMP accumulation was measured. Cardiac membranes from WKY rats (Harlan Bioproducts for Science) were pelleted and incubated in a cAMP reaction buffer to determine cAMP accumulation in the presence or absence of fenoterol and PTX. The measurement of cAMP accumulation in the presence of PTX is indicative of G_s activation. Fenoterol induced maximal cAMP accumulation of approximately 250% of control in the absence of PTX. The presence of PTX did not affect cAMP accumulation induced by fenoterol over a wide range of concentrations (10^{-8}M to 10^{-4}M). These results indicate that fenoterol selectively activates the β_2AR-coupled G_s signaling pathway.

Example 4

This example demonstrates that fenoterol selectively activates the β_2AR-coupled G_s signaling pathway in intact cells isolated from failing hearts.

Spontaneous hypertensive rats (SHR; Harlan Bioproducts for Science, Indianapolis, IN; Pfeffer et al., Am. J. Physiol. 237: H461 (1979); and Conrad et al., Am. J. Physiol. 260: H136 (1991)), which represent an animal model of cardiovascular disease, in particular chronic hypertensive heart disease and heart failure, were studied at an age of 18-24 months, when they demonstrate cardiac hypertrophy and physical signs of heart failure (e.g., resting tachycardia, tachypnea, and pleural and/or pericardial effusions; Pfeffer et al., Am. J. Physiol. 237: H461 (1979); and Conrad et al., Am. J. Physiol. 260: H136 (1991)) and were compared to age-matched Wistar-Kyoto rats (WKY; Harlan Bioproducts for Science) as a control. Blood pressures were measured weekly in every animal by the tail-cuff method in conscious non-sedated animals pre-warmed to 37 °C. At three months of age, SHR were clearly hypertensive and had systolic blood pressures of 182 ± 21 mm Hg (vs. WKY: 121 ± 11 mm Hg, $p < 0.05$, $n = 21$). Cardiac hypertrophy was confirmed by significant increases in heart weight (wet)/ body weight (mg/g; SHR: 5.10 ± 0.8 vs. WKY: 3.8 ± 0.5, $p < 0.05$, $n = 13$), echocardiographic measurements of left ventricular wall thickness (e.g., posterior wall thickness, mm; SHR: 2.1 ± 0.3 vs. WKY: 1.6 ± 0.3, $p < 0.05$, $n = 10$), electrically determined measurements of cell capacitance (pF; SHR: 270 ± 43 vs. WKY 180 ± 30, $p < 0.05$, $n = 44$), and cross-sectional area of cells computed from images of transmitted light microscopy (μm^2, SHR: 296 ± 78 vs. WKY: 135 ± 58, $p < 0.001$, $n = 256$).

β_2AR stimulation by fenoterol enhanced the amplitude of contraction in SHR to an extent similar to that observed in age-matched WKY.

Example 5

This example demonstrates that fenoterol selectively activates the β_2AR-coupled G_s signaling pathway in intact cells from failing hearts.

Six week old Dahl salt-sensitive (DS; SS/JrHsd) and salt-resistant (DR; SR/JrHsd) rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) were given normal (0.2 %) or high (8 %) NaCl diets (ICN Pharmaceuticals, Costa Mesa, CA). Starting from week 4 of the high NaCl diet, the rats were given 8 % NaCl diets five days per week and 0.2 % NaCl diets two days per week (days 5 and 7). At baseline, systolic blood press (SBP) in 12 DS and 12 DR was 106 ± 2 and 108 ± 2 mm Hg, respectively. In DS on 8 % NaCl diet, SBP gradually rose and reached a plateau on week 2 (161 ± 7 mm Hg, $n = 6$, $p < 0.001$ vs. baseline). In DR after 2 weeks of 8 %

NaCl diet SBP was 118 ± 4 mm Hg (n=6, p <0.001 vs. DS at week 2). SBP in DS after 2 weeks of 0.2 % NaCl diet was 114 ± 3 mm Hg (n = 6, p < 0.001 vs. DS on 8 % NaCl diet). On week 8 of high NaCl diet, DS exhibited symptoms of congestive heart failure, i.e., rapid and labored respiration, loss of physical activity and decrease of body weight. When sacrificed, pathological examination revealed pleural effusion and ascites. The heart/body weight ratios in DS on 8 % and 0.2 % NaCl diets were 6.9 ± 0.9 and 3.7 ± 0.1 (p < 0.01). The lung/body weight ratios were 11.4 ± 0.8 and 4.1 ± 0.3 (p < 0.01), respectively. In DR on 8 % NaCl diet, heart/body and lung/body weight ratios were 4.0 ± 0.1 and 3.9 ± 0.1 (p < 0.01 vs. DS on 8 % diet for both parameters).

Contractile response to fenoterol remained intact in DS myocytes relative to age-matched DR myocytes.

All of the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be apparent to those of ordinary skill in the art that variations in the preferred embodiments can be prepared and used and that the invention can be practiced otherwise than as specifically described herein. The present invention is intended to include such variations and alternative practices. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of using a β_2 adrenergic receptor agonist that selectively activates G_s proteins in the treatment of cardiovascular disease, which method comprises administering to a mammal in need thereof a treatment effective amount of the β_2 adrenergic receptor agonist so as to treat cardiovascular disease.

2. The method of claim 1, wherein said cardiovascular disease is acute heart failure.

3. The method of claim 1, wherein said cardiovascular disease is chronic heart failure.

4. The method of claim 1, wherein said cardiovascular disease is aging heart.

5. The method of any of claims 1-4, wherein said β_2 adrenergic receptor agonist is fenoterol or an acid additional salt thereof.

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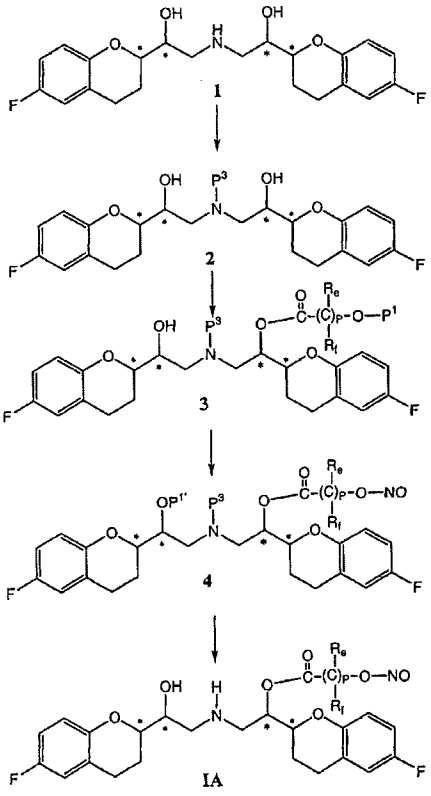
[Continued on next page]

(54) Title: NITROSATED AND NITROSYLATED NEBIVOLOL AND ITS METABOLITES, COMPOSITIONS AND METHODS OF USE



(57) Abstract: The invention describes novel nitrosated and/or nitrosylated nebivolol, novel nitrosated and/or nitrosylated metabolites of nebivolol and novel compositions comprising at least one nitrosated and/or nitrosylated nebivolol and/or at least one nitrosated and/or nitrosylated metabolite of nebivolol, and optionally, at least one nitric oxide donor and/or at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one compound used to treat cardiovascular disease or a pharmaceutically acceptable salt thereof, and/or at least one nitrosated compound used to treat cardiovascular diseases. The invention also provides novel compositions comprising nebivolol and/or at least one metabolite of nebivolol and at least one nitric oxide donor, and, optionally, at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one compound used to treat cardiovascular diseases or a pharmaceutically acceptable salt thereof, and/or at least one nitrosated compound used to treat cardiovascular diseases. The compounds and compositions of the invention can also be bound to a matrix. The nitric oxide donor is a compound that donates, transfers or releases nitric oxide, elevates endogenous levels of endothelium-derived relaxing factor, stimulates endogenous synthesis of nitric oxide or is a substrate for nitric oxide synthase and may preferably be isosorbide dinitrate and/or isosorbide mononitrate. The antioxidant may preferably be a hydralazine compound or a pharmaceutically acceptable salt thereof. The invention also provides methods for treating and/or preventing vascular diseases characterized by nitric oxide insufficiency; and for treating and/or preventing Raynaud's syndrome; and for treating and/or preventing cardiovascular diseases or disorders.

WO 02/087508 A2

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NITROSATED AND NITROSYLATED NEBIVOLOL AND ITS METABOLITES, COMPOSITIONS AND METHODS OF USE

RELATED APPLICATIONS

This application claims priority to U. S. Provisional Application No. 60/287,725 filed May 2, 2001.

FIELD OF THE INVENTION

The invention describes novel nitrosated and/or nitrosylated nebivolol, novel nitrosated and/or nitrosylated metabolites of nebivolol and novel compositions comprising at least one nitrosated and/or nitrosylated nebivolol and/or at least one nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, at least one nitric oxide donor and/or at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one compound used to treat cardiovascular diseases or a pharmaceutically acceptable salt thereof, and/or at least one nitrosated compound used to treat cardiovascular diseases. The invention also provides novel compositions comprising nebivolol and/or at least one metabolite of nebivolol and at least one nitric oxide donor, and, optionally, at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one compound used to treat cardiovascular diseases or a pharmaceutically acceptable salt thereof, and/or at least one nitrosated compound used to treat cardiovascular diseases. The compounds and compositions of the invention can also be bound to a matrix. The nitric oxide donor is a compound that donates, transfers or releases nitric oxide, elevates endogenous levels of endothelium-derived relaxing factor, stimulates endogenous synthesis of nitric oxide or is a substrate for nitric oxide synthase and may preferably be isosorbide dinitrate and/or isosorbide mononitrate. The antioxidant may preferably be a hydralazine compound or a pharmaceutically acceptable salt thereof. The invention also provides methods for treating and/or preventing vascular diseases characterized by nitric oxide insufficiency; and for treating and/or preventing Raynaud's syndrome; and for treating and/or preventing cardiovascular diseases or disorders.

BACKGROUND OF THE INVENTION

The decline in cardiovascular morbidity and mortality in the United States over the past three decades has been the result of significant advances in research on cardiovascular disease mechanisms and therapeutic strategies. The incidence and prevalence of myocardial infarction and death from myocardial infarction, as well as that from cerebrovascular accident, have decreased significantly over this period largely owing to

advances in prevention, early diagnosis, and treatment of these very common diseases.

Analysis of outcomes by race, however, paints quite a different picture: life expectancy and cardiovascular morbidity rates have improved far less for blacks than whites. Available data show that the likelihood of dying from cardiovascular disease is far greater among black Americans than among white Americans. In this decade, the death rate from cardiovascular disease for black males was 353 per 100,000 population, while that for white males was 244 per 100,000; the rate for black females was 226 per 100,000; while that for white females was 135 per 100,000. Consonant with this important demographic parameter is the observation that there is a higher prevalence of several of the important risk factors for cardiovascular disease, e.g., hypertension, smoking, diabetes mellitus, obesity, and left ventricular hypertrophy, among blacks compared with whites. In addition, outcomes of cardiovascular events are worse for blacks than whites. Following myocardial infarction, blacks have a 50% higher annual mortality rate than whites, and their five-year survival is only 70%. Thus, the many advances in cardiovascular medicine that account for the overall improvement in cardiovascular health in the general population has failed to translate into comparable racial benefits.

There is a need in the art for new and more effective compositions and methods for treating vascular diseases. The invention is directed to these, as well as other, important ends.

SUMMARY OF THE INVENTION

The invention describes novel nitrosated and/or nitrosylated nebivolol, novel nitrosated and/or nitrosylated metabolites of nebivolol and methods of treating and/or preventing vascular diseases characterized by nitric oxide insufficiency, and Raynaud's syndrome by administering at least one nitrosated and/or nitrosylated nebivolol and/or at least one nitrosated and/or nitrosylated metabolite of nebivolol that is capable of releasing a therapeutically effective amount of nitric oxide to a targeted site effected by the vascular disease.

One embodiment of the invention provides novel nitrosated and/or nitrosylated nebivolol and/or novel nitrosated and/or nitrosylated metabolites of nebivolol. The nebivolol and/or its metabolites can be nitrosated and/or nitrosylated through one or more sites such as oxygen (hydroxyl condensation), sulfur (sulfhydryl condensation) and/or nitrogen. The invention also provides compositions comprising a therapeutically effective amount of such compounds in a pharmaceutically acceptable carrier.

Another embodiment of the invention provides compositions comprising a therapeutically effective amount of nebivolol that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and at least one compound that donates, transfers or releases nitrogen monoxide as a charged species, i.e., nitrosonium (NO^+) or nitroxyl (NO-), or as the neutral species, nitric oxide (NO•), and/or stimulates endogenous production of nitric oxide or EDRF *in vivo* and/or is a substrate for nitric oxide synthase. The nitric oxide donor may preferably be isosorbide dinitrate and/or isosorbide mononitrate. The invention also provides for such compositions in a pharmaceutically acceptable carrier.

Yet another embodiment of the invention provides compositions comprising a therapeutically effective of nebivolol that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and at least one antioxidant, and, optionally, at least one compound that donates, transfers or releases nitrogen monoxide as a charged species, i.e., nitrosonium (NO^+) or nitroxyl (NO-), or as the neutral species, nitric oxide (NO•), and/or stimulates endogenous production of nitric oxide or EDRF *in vivo* and/or is a substrate for nitric oxide synthase. The antioxidant may preferably be a hydralazine compound or a pharmaceutically acceptable salt thereof. The nitric oxide donor may preferably be isosorbide dinitrate and/or isosorbide mononitrate. The invention also provides for such compositions in a pharmaceutically acceptable carrier.

Another embodiment of the invention provides compositions comprising a therapeutically effective amount of nebivolol that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and, optionally, at least one antioxidant, and/or at least one compound that donates, transfers or releases nitrogen monoxide as a charged species, i.e., nitrosonium (NO^+) or nitroxyl (NO-), or as the neutral species, nitric oxide (NO•), and/or stimulates endogenous production of nitric oxide or EDRF *in vivo* and/or is a substrate for nitric oxide synthase and/or at least one compound used to treat cardiovascular diseases, optionally substituted with at least one NO_2 group (i.e., nitrosated). The antioxidant may preferably be a hydralazine compound or a pharmaceutically acceptable salt thereof. The

nitric oxide donor may preferably be isosorbide dinitrate and/or isosorbide mononitrate. The invention also provides for such compositions in a pharmaceutically acceptable carrier.

The invention provides methods for treating and/or preventing vascular diseases characterized by nitric oxide insufficiency by administering to a patient a therapeutically effective amount of nebivolol that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group (i.e. nitrosylated and/or nitrosated), and, optionally, at least one compound that donates, transfers or releases nitric oxide, elevates endogenous levels of endothelium-derived relaxing factor, stimulates endogenous synthesis of nitric oxide or is a substrate for nitric oxide synthase, and/or at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one compound used to treat cardiovascular diseases, or a pharmaceutically acceptable salt thereof , optionally substituted with at least one NO_2 group (i.e., nitrosated). The nitric oxide donor may preferably be isosorbide dinitrate and/or isosorbide mononitrate. The antioxidant may preferably be a hydralazine compound or a pharmaceutically acceptable salt thereof. The nebivolol and/or the metabolite of nebivolol and optional nitric oxide donor compound, antioxidant, and/or compound used to treat cardiovascular diseases can be administered separately or as components of the same composition in one or more pharmaceutically acceptable carriers.

In another embodiment, the invention provides methods for treating and/or preventing Raynaud's syndrome by administering to a patient a therapeutically effective amount of nebivolol that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and, optionally, at least one compound that donates, transfers or releases nitric oxide, elevates endogenous levels of endothelium-derived relaxing factor, stimulates endogenous synthesis of nitric oxide or is a substrate for nitric oxide synthase, and/or at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one compound used to treat cardiovascular diseases that is optionally substituted with at least one NO_2 group (i.e., nitrosated). The nitric oxide donor may preferably be isosorbide dinitrate and/or isosorbide mononitrate. The antioxidant may preferably be a hydralazine compound or a pharmaceutically acceptable salt thereof. The nebivolol and/or metabolite of nebivolol and optional nitric oxide donor compound, antioxidant, and/or compound used to treat

cardiovascular diseases can be administered separately or as components of the same composition in one or more pharmaceutically acceptable carriers.

Another embodiment of the invention describes compositions and methods for making compositions comprising nebivolol that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated), and, optionally, at least one compound that donates, transfers or releases nitric oxide and/or stimulates endogenous production of nitric oxide or EDRF *in vivo* and/or is a substrate for nitric oxide synthase, that are bound to a natural or synthetic matrix, which can be applied with specificity to a biological site of interest. For example, the matrix containing the nitrosated and/or nitrosylated nebivolol can be used to coat the surface of a medical device or instrument that comes into contact with blood (including blood components, blood products and the like) or vascular tissue.

Another embodiment of the invention also provides methods for administering to a patient in need thereof a therapeutically effective amount of nebivolol and/or at least one metabolite of nebivolol and at least one compound that donates, transfers or releases nitric oxide as a charged species, i.e., nitrosonium (NO^+) or nitroxyl (NO-), or as the neutral species, nitric oxide (NO•), and/or stimulates endogenous production of nitric oxide or EDRF *in vivo* and/or is a substrate for nitric oxide synthase for treating and/or preventing cardiovascular diseases or disorders. The methods can further comprise administering a therapeutically effective amount of at least one therapeutic agent. Alternatively, the methods for treating and/or preventing cardiovascular diseases or disorders, can comprise administering a therapeutically effective amount of at nebivolol and/or at least one metabolite of nebivolol, at least one therapeutic agent, and, optionally, at least one compound that donates, transfers or releases nitric oxide as a charged species, i.e., nitrosonium (NO^+) or nitroxyl (NO-), or as the neutral species, nitric oxide (NO•), and/or stimulates endogenous production of nitric oxide or EDRF *in vivo* and/or is a substrate for nitric oxide synthase. The nebivolol, the metabolite of nebivolol, the nitric oxide donors, and the therapeutic agents can be administered separately or as components of the same composition in one or more pharmaceutically acceptable carriers.

Yet another embodiment of the invention describes methods for the prevention of platelet aggregation and platelet adhesion caused by the exposure of blood to a medical device or instrument by incorporating at least one nitrosated and/or nitrosylated nebivolol

and/or at least one metabolite of nebivolol that is capable of releasing a therapeutically effective amount of nitric oxide into and/or on the portion(s) of the medical device that come into contact with blood (including blood components and blood products) or vascular tissue. The methods can further comprise incorporating at least one compound that donates, transfers or releases nitric oxide, and/or stimulates endogenous production of nitric oxide or EDRF *in vivo* and/or is a substrate for nitric oxide synthase, and, optionally, at least one therapeutic agent into and/or on the portion(s) of the medical device that come into contact with blood or vascular tissue. Alternatively the methods can comprise incorporating nebivolol and/or at least one metabolite of nebivolol and at least one NO donor, and, optionally, at least one therapeutic agent into and/or on the portion(s) of the medical device that comes into contact with blood or vascular tissue.

Another embodiment of the invention relates to the local administration of nebivolol that is optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group, and, optionally, at least one therapeutic agent and/or at least one nitric oxide donor, to treat injured tissue, such as damaged blood vessels.

These and other aspects of the invention are described in detail herein.

The following drawings are illustrative of embodiments of the invention and do not limit the scope of the invention as defined by the claims.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is the synthetic scheme for the preparation of nitrite containing compounds of Formula (I).

Fig. 2 is the synthetic scheme for the preparation of nitrosothiol containing compounds of Formula (I).

Fig. 3 is the synthetic scheme for the preparation of nitrate containing compounds of Formula (I).

Fig. 4 is the synthetic scheme for the preparation of 2-hydroxy-2- nitrosohydrazine containing compounds of Formula (I).

Fig. 5 is the synthetic scheme for the preparation of nitrite containing compounds of Formula (II).

Fig. 6 is the synthetic scheme for the preparation of nitrosothiol containing compounds of Formula (II).

Fig. 7 is the synthetic scheme for the preparation of nitrate containing compounds of

Formula (II).

Fig. 8 is the synthetic scheme for the preparation of 2-hydroxy-2- nitrosohydrazine containing compounds of Formula (II).

Fig. 9 is the synthetic scheme for the preparation of nitrite containing compounds of Formula (III).

Fig. 10 is the synthetic scheme for the preparation of nitrite containing compounds of Formula (III).

Fig. 11 is the synthetic scheme for the preparation of nitrosothiol containing compounds of Formula (III).

Fig. 12 is the synthetic scheme for the preparation of nitrosothiol containing compounds of Formula (III).

Fig. 13 is the synthetic scheme for the preparation of nitrate containing compounds of Formula (III).

Fig. 14 is the synthetic scheme for the preparation of nitrate containing compounds of Formula (III).

Fig. 15 is the synthetic scheme for the preparation of 2-hydroxy-2- nitrosohydrazine containing compounds of Formula (III).

Fig. 16 is the synthetic scheme for the preparation of 2-hydroxy-2- nitrosohydrazine containing compounds of Formula (III).

DETAILED DESCRIPTION OF THE INVENTION

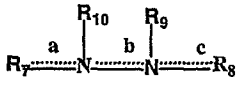
As used throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings.

"Patient" refers to animals, preferably mammals, most preferably humans, and includes males and females.

"Therapeutically effective amount" refers to the amount of the compound and/or composition that is effective to achieve its intended purpose.

"Hydrazino" refers to H_2N-N(H)-.

"Hydralazine compound" refers to a compound having the Formula (VI):



VI

wherein a, b and c are independently a single or double bond; R_7 and R_8 are each

independently a hydrogen, an alkyl, an ester or a heterocyclic ring; R_9 and R_{10} are each independently a lone pair of electrons or a hydrogen, with the proviso that at least one of R_7, R_8, R_9 and R_{10} is not a hydrogen. Exemplary hydralazine compounds include budralazine, cadralazine, dihydralazine, endralazine, hydralazine, pildralazine, todralazine, and the like.

"Compound used to treat cardiovascular diseases" refers to any therapeutic compound, or a pharmaceutically acceptable salt thereof, used to treat any cardiovascular disease.

"Vascular diseases characterized by nitric oxide insufficiency" include, but are not limited to, cardiovascular diseases; diseases resulting from oxidative stress; hypertension (e.g., low-renin hypertension; salt-sensitive hypertension; low-renin, salt-sensitive hypertension; primary pulmonary hypertension; thromboembolic pulmonary hypertension; pregnancy-induced hypertension; renovascular hypertension, hypertension-dependent end-stage renal disease), heart failure (e.g., microvascular cardiac ischemia), and left ventricular hypertrophy with disproportionate microvascularization, (i.e., inadequate vascularity) or diastolic dysfunction.

"Cardiovascular diseases" refers to any cardiovascular disease or disorder known in the art, including, but not limited to, congestive heart failure, hypertension, pulmonary hypertension, myocardial and cerebral infarctions, atherosclerosis, atherogenesis, thrombosis, ischemic heart disease, post-angioplasty restenosis, coronary artery diseases, renal failure, stable, unstable and variant (Prinzmetal) angina, cardiac edema, renal insufficiency, nephrotic edema, hepatic edema, stroke, transient ischemic attacks, cerebrovascular accidents, restenosis, controlling blood pressure in hypertension (especially hypertension associated with cardiovascular surgical procedures), platelet adhesion, platelet aggregation, smooth muscle cell proliferation, pulmonary edema associated with acute myocardial infarction, vascular complications associated with the use of medical devices, wounds associated with the use of medical devices, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia, bleeding disorders, and the like. Complications associated with the use of medical devices may occur as a result of increased platelet deposition, activation, thrombus formation or consumption of platelets and coagulation proteins. Such complications, which are within the definition of "cardiovascular disease or disorder," include, for example, myocardial infarction, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia, bleeding disorders and/or any other complications which occur either directly or indirectly as

a result of the foregoing disorders.

"Restenosis" is a cardiovascular disease or disorder that refers to the closure of a peripheral or coronary artery following trauma to the artery caused by an injury such as, for example, angioplasty, balloon dilation, atherectomy, laser ablation treatment or stent insertion. For these angioplasty procedures, restenosis occurs at a rate of about 30-60% depending upon the vessel location, lesion length and a number of other variables. Restenosis can also occur following a number of invasive surgical techniques, such as, for example, transplant surgery, vein grafting, coronary artery bypass surgery, endarterectomy, heart transplantation, ballon angioplasty, atherectomy, laser ablation, endovascular stenting, and the like.

"Atherosclerosis" is a form of chronic vascular injury in which some of the normal vascular smooth muscle cells in the artery wall, which ordinarily control vascular tone regulating blood flow, change their nature and develop "cancer-like" behavior. These vascular smooth muscle cells become abnormally proliferative, secreting substances such as growth factors, tissue-degradation enzymes and other proteins, which enable them to invade and spread into the inner vessel lining, blocking blood flow and making that vessel abnormally susceptible to being completely blocked by local blood clotting, resulting in the death of the tissue served by that artery.

"Diseases resulting from oxidative stress" refers to any disease that involves the generation of free radicals or radical compounds, such as, for example, atherogenesis, atheromatosis, arteriosclerosis, artherosclerosis, vascular hypertrophy associated with hypertension, hyperlipoproteinaemia, normal vascular degeneration through aging, parathyroidal reactive hyperplasia, chronic renal disease, neoplastic diseases, inflammatory diseases, neurological and acute bronchopulmonary disease, tumorigenesis, ischemia-reperfusion syndrome, arthritis, sepsis, and the like.

"Therapeutic agent" includes any therapeutic agent that can inhibit the cellular activity of a vascular smooth muscle cell, for example, proliferation, migration, increase in cell volume, increase in extracellular matrix synthesis (e.g., collagens, proteoglycans, and the like), or secretion of extracellular matrix materials by the cell, biologically stenting a vessel and/or reduce or inhibit vascular remodeling and/or inhibit or reduce vascular smooth muscle proliferation following a procedural vascular trauma. Although nitric oxide donors have therapeutic activity, the term "therapeutic agent" does not include the nitric oxide donors described herein, since nitric oxide donors are separately defined.

"Artificial surface" refers to any natural or synthetic material contained in a device or apparatus that is in contact with blood, vasculature or other tissues.

"Blood" includes blood products, blood components and the like.

"Platelet adhesion" refers to the contact of a platelet with a foreign surface, including any artificial surface, such as a medical device or instrument, as well as an injured vascular surfaces, such as collagen. Platelet adhesion does not require platelet activation. Unactivated, circulating platelets will adhere to injured vascular surfaces or artificial surfaces via binding interactions between circulating von Willdebrand factor and platelet surface glycoprotein Ib/IX.

"Platelet aggregation" refers to the binding of one or more platelets to each other. Platelet aggregation is commonly referred to in the context of generalized atherosclerosis, not with respect to platelet adhesion on vasculature damaged as a result of physical injury during a medical procedure. Platelet aggregation requires platelet activation, which depends on the interaction between the ligand and its specific platelet surface receptor.

"Platelet activation" refers either to the change in conformation (shape) of a cell, expression of cell surface proteins (e.g., the IIb/IIIa receptor complex, loss of GPIb surface protein), and secretion of platelet derived factors (e.g., serotonin, growth factors).

"Passivation" refers to the coating of a surface, which renders the surface non-reactive.

"Medical device" refers to any intravascular or extravascular medical devices, medical instruments, foreign bodies and the like. Examples of intravascular medical devices and instruments include balloons or catheter tips adapted for insertion, prosthetic heart valves, sutures, synthetic vessel grafts, stents (e.g. Palmaz-Schatz stent), drug pumps, arteriovenous shunts, artificial heart valves, artificial implants, foreign bodies introduced surgically into the blood vessels or at vascular sites, leads, pacemakers, implantable pulse generators, implantable cardiac defibrillators, cardioverter defibrillators, defibrillators, spinal stimulators, brain stimulators, sacral nerve stimulators, chemical sensors, and the like. Examples of extravascular medical devices and instruments include plastic tubing, dialysis bags or membranes whose surfaces come in contact with the blood stream of a patient.

"Transdermal" refers to the delivery of a compound by passage through the skin and into the blood stream.

"Transmucosal" refers to delivery of a compound by passage of the

compound through the mucosal tissue and into the blood stream.

"Penetration enhancement" or "permeation enhancement" refers to an increase in the permeability of the skin or mucosal tissue to a selected pharmacologically active compound such that the rate at which the compound permeates through the skin or mucosal tissue is increased.

"Carriers" or "vehicles" refers to carrier materials suitable for compound administration and include any such material known in the art such as, for example, any liquid, gel, solvent, liquid diluent, solubilizer, or the like, which is non-toxic and which does not interact with any components of the composition in a deleterious manner.

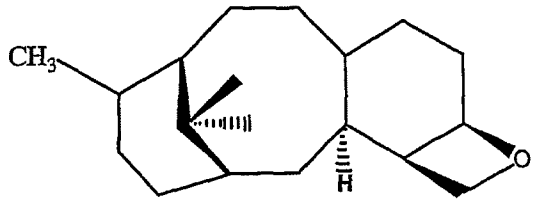
"Sustained release" refers to the release of a therapeutically active compound and/or composition such that the blood levels of the therapeutically active compound are maintained within a desirable therapeutic range over an extended period of time. The sustained release formulation can be prepared using any conventional method known to one skilled in the art to obtain the desired release characteristics.

"Nitric oxide adduct" or "NO adduct" refers to compounds and functional groups which, under physiological conditions, can donate, release and/or directly or indirectly transfer any of the three redox forms of nitrogen monoxide (NO^+, NO^-, NO•), such that the biological activity of the nitrogen monoxide species is expressed at the intended site of action.

"Nitric oxide releasing" or "nitric oxide donating" refers to methods of donating, releasing and/or directly or indirectly transferring any of the three redox forms of nitrogen monoxide (NO^+, NO^-, NO•), such that the biological activity of the nitrogen monoxide species is expressed at the intended site of action.

"Nitric oxide donor" or "NO donor" refers to compounds that donate, release and/or directly or indirectly transfer a nitrogen monoxide species, and/or stimulate the endogenous production of nitric oxide or endothelium-derived relaxing factor (EDRF) *in vivo* and/or elevate endogenous levels of nitric oxide or EDRF *in vivo*. "NO donor" also includes compounds that are substrates for nitric oxide synthase.

"Taxane" refers to any compound that contains the carbon core framework represented by Formula A:



A

"Alkyl" refers to a lower alkyl group, a haloalkyl group, a hydroxyalkyl group, an alkenyl group, an alkynyl group, a bridged cycloalkyl group, a cycloalkyl group or a heterocyclic ring, as defined herein. An alkyl group may also comprise one or more radical species, such as, for example a cycloalkylalkyl group or a heterocyclicalkyl group.

"Lower alkyl" refers to branched or straight chain acyclic alkyl group comprising one to about ten carbon atoms (preferably one to about eight carbon atoms, more preferably one to about six carbon atoms). Exemplary lower alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, t-butyl, pentyl, neopentyl, iso-amyl, hexyl, octyl, and the like.

"Substituted lower alkyl" refers to a lower alkyl group, as defined herein, wherein one or more of the hydrogen atoms have been replaced with one or more R^{100} groups, wherein each R^{100} is independently a hydroxy, an oxo, a carboxyl, a carboxamido, a halo, a cyano or an amino group, as defined herein.

"Haloalkyl" refers to a lower alkyl group, an alkenyl group, an alkynyl group, a bridged cycloalkyl group, a cycloalkyl group or a heterocyclic ring, as defined herein, to which is appended one or more halogens, as defined herein. Exemplary haloalkyl groups include trifluoromethyl, chloromethyl, 2-bromobutyl, 1-bromo-2-chloro-pentyl, and the like.

"Alkenyl" refers to a branched or straight chain C_2-C_{10} hydrocarbon (preferably a C_2-C_8 hydrocarbon, more preferably a C_2-C_6 hydrocarbon) that can comprise one or more carbon-carbon double bonds. Exemplary alkenyl groups include propylenyl, buten-1-yl, isobutenyl, penten-1-yl, 2,2-methylbuten-1-yl, 3-methylbuten-1-yl, hexan-1-yl, hepten-1-yl, octen-1-yl, and the like.

"Lower alkenyl" refers to a branched or straight chain C_2-C_4 hydrocarbon that can comprise one or two carbon-carbon double bonds.

"Substituted alkenyl" refers to a branched or straight chain C_2-C_{10} hydrocarbon (preferably a C_2-C_8 hydrocarbon, more preferably a C_2-C_6 hydrocarbon) which can comprise one or more carbon-carbon double bonds, wherein one or more of the hydrogen atoms have been replaced with one or more R^{100} groups, wherein each R^{100} is independently a hydroxy, an oxo, a carboxyl, a carboxamido, a halo, a cyano or an amino group, as defined herein.

"Alkynyl" refers to an unsaturated acyclic C_2-C_{10} hydrocarbon (preferably a C_2-C_8 hydrocarbon, more preferably a C_2-C_6 hydrocarbon) that can comprise one or more carbon-carbon triple bonds. Exemplary alkynyl groups include ethynyl, propynyl, butyn-1-yl, butyn-2-yl, pentyl-1-yl, pentyl-2-yl, 3-methylbutyn-1-yl, hexyl-1-yl, hexyl-2-yl, hexyl-3-yl, 3,3-dimethyl-butyn-1-yl, and the like.

"Bridged cycloalkyl" refers to two or more saturated or unsaturated cycloalkyl groups, saturated or unsaturated heterocyclic groups, or a combination thereof fused via adjacent or non-adjacent atoms. Bridged cycloalkyl groups can be unsubstituted or substituted with one, two or three substituents independently selected from alkyl, alkoxy, amino, alkylamino, dialkylamino, hydroxy, halo, carboxyl, alkylcarboxylic acid, aryl, amidyl, ester, alkylcarboxylic ester, carboxamido, alkylcarboxamido, oxo and nitro. Exemplary bridged cycloalkyl groups include adamantyl, decahydronapthyl, quinuclidyl, 2,6-dioxabicyclo(3.3.0)octane, 7-oxabycyclo(2.2.1)heptyl, 8-azabicyclo(3,2,1)oct-2-enyl and the like.

"Cycloalkyl" refers to a saturated or unsaturated cyclic hydrocarbon comprising from about 3 to about 10 carbon atoms. Cycloalkyl groups can be unsubstituted or substituted with one, two or three substituents independently selected from alkyl, alkoxy, amino, alkylamino, dialkylamino, arylamino, diarylamino, alkylarylamino, aryl, amidyl, ester, hydroxy, halo, carboxyl, alkylcarboxylic acid, alkylcarboxylic ester, carboxamido, alkylcarboxamido, oxo, alkylsulfinyl, and nitro. Exemplary cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclohexenyl, cyclohepta-1,3-dienyl, and the like.

"Heterocyclic ring or group" refers to a saturated or unsaturated cyclic hydrocarbon group having about 2 to about 10 carbon atoms (preferably about 4 to about 6 carbon atoms) where 1 to about 4 carbon atoms are replaced by one or more nitrogen, oxygen and/or sulfur atoms. Sulfur maybe in the thio, sulfinyl or sulfonyl oxidation state. The heterocyclic ring or group can be fused to an aromatic hydrocarbon group. Heterocyclic groups can be

unsubstituted or substituted with one, two or three substituents independently selected from alkyl, alkoxy, amino, alkylthio, aryloxy, arylthio, arylalkyl, hydroxy, oxo, thial, halo, carboxyl, carboxylic ester, alkylcarboxylic acid, alkylcarboxylic ester, aryl, arylcarboxylic acid, arylcarboxylic ester, amidyl, ester, alkylcarbonyl, arylcarbonyl, alkylsulfinyl, carboxamido, alkylcarboxamido, arylcarboxamido, sulfonic acid, sulfonic ester, sulfonamido and nitro. Exemplary heterocyclic groups include pyrrolyl, furyl, thienyl, 3-pyrrolinyl,4,5,6-trihydro-2H-pyranyl, pyridinyl, 1,4-dihydropyridinyl, pyrazolyl, triazolyl, pyrimidinyl, pyridazinyl, oxazolyl, thiazolyl, imidazolyl, indolyl, thiophenyl, furanyl, tetrhydrofuranyl, tetrazolyl, pyrrolinyl, pyrrolindinyl, oxazolindinyl 1,3-dioxolanyl, imidazolinyl, imidazolindinyl, pyrazolinyl, pyrazolidinyl, isoxazolyl, isothiazolyl, 1,2,3-oxadiazolyl, 1,2,3-triazolyl, 1,3,4-thiadiazolyl, 2H-pyranyl, 4H-pyranyl, piperidinyl, 1,4-dioxanyl, morpholinyl, 1,4-dithianyl, thiomorpholinyl, pyrazinyl, piperazinyl, 1,3,5-triazinyl, 1,3,5-trithianyl, benzo(b)thiophenyl, benzimidazolyl, benzothiazolinyl, quinolinyl, and the like.

"Heterocyclic compounds" refer to mono- and polycyclic compounds comprising at least one aryl or heterocyclic ring.

"Aryl" refers to a monocyclic, bicyclic, carbocyclic or heterocyclic ring system comprising one or two aromatic rings. Exemplary aryl groups include phenyl, pyridyl, napthyl, quinoyl, tetrahydronaphthyl, furanyl, indanyl, indenyl, indoyl, and the like. Aryl groups (including bicyclic aryl groups) can be unsubstituted or substituted with one, two or three substituents independently selected from alkyl, alkoxy, alkylthio, amino, alkylamino, dialkylamino, arylamino, diarylamino, alkylarylamino, halo, cyano, alkylsulfinyl, hydroxy, carboxyl, carboxylic ester, alkylcarboxylic acid, alkylcarboxylic ester, aryl, arylcarboxylic acid, arylcarboxylic ester, alkylcarbonyl, arylcarbonyl, amidyl, ester, carboxamido, alkylcarboxamido, carbomyl, sulfonic acid, sulfonic ester, sulfonamido and nitro. Exemplary substituted aryl groups include tetrafluorophenyl, pentafluorophenyl, sulfonamide, alkylsulfonyl, arylsulfonyl, and the like.

"Cycloalkenyl" refers to an unsaturated cyclic C_2-C_{10} hydrocarbon (preferably a C_2-C_8 hydrocarbon, more preferably a C_2-C_6 hydrocarbon) which can comprise one or more carbon-carbon triple bonds.

"Arylalkyl" refers to an aryl radical, as defined herein, attached to an alkyl radical, as defined herein. Exemplary arylalkyl groups include benzyl, phenylethyl, 4-hydroxybenzyl, 3-fluorobenzyl, 2-fluorophenylethyl, and the like.

"Alkylaryl" refers to an alkyl group, as defined herein, to which is appended an aryl group, as defined herein. Exemplary alkylaryl groups include benzyl, phenylethyl, hydroxybenzyl, fluorobenzyl, fluorophenylethyl, and the like.

"Arylalkenyl" refers to an aryl radical, as defined herein, attached to an alkenyl radical, as defined herein. Exemplary arylalkenyl groups include styryl, propenylphenyl, and the like.

"Cycloalkylalkyl" refers to a cycloalkyl radical, as defined herein, attached to an alkyl radical, as defined herein.

"Cycloalkylalkoxy" refers to a cycloalkyl radical, as defined herein, attached to an alkoxy radical, as defined herein.

"Cycloalkylalkylthio" refers to a cycloalkyl radical, as defined herein, attached to an alkylthio radical, as defined herein.

"Heterocyclicalkyl" refers to a heterocyclic ring radical, as defined herein, attached to an alkyl radical, as defined herein.

"Arylheterocyclic ring" refers to a bi- or tricyclic ring comprised of an aryl ring, as defined herein, appended via two adjacent carbon atoms of the aryl ring to a heterocyclic ring, as defined herein. Exemplary arylheterocyclic rings include dihydroindole, 1,2,3,4-tetra-hydroquinoline, and the like.

"Alkoxy" refers to $R_{50}O$-, wherein R_{50} is an alkyl group, as defined herein (preferably a lower alkyl group or a haloalkyl group, as defined herein). Exemplary alkoxy groups include methoxy, ethoxy, t-butoxy, cyclopentyloxy, trifluoromethoxy, and the like.

"Lower alkoxy" refers to a lower alkyl group, as defined herein, appended to an oxygen atom.

"Aryloxy" refers to $R_{55}O$-, wherein R_{55} is an aryl group, as defined herein. Exemplary arylkoxy groups include napthyloxy, quinolyloxy, isoquinolizinyloxy, and the like.

"Alkylthio" refers to $R_{50}S$-, wherein R_{50} is an alkyl group, as defined herein.

"Lower alkylthio" refers to a lower alkyl group, as defined herein, appended to a thio group, as defined herein.

"Arylalkoxy" or "alkoxyaryl" refers to an alkoxy group, as defined herein, to which is appended an aryl group, as defined herein. Exemplary arylalkoxy groups include benzyloxy, phenylethoxy, chlorophenylethoxy, and the like.

"Alkoxyalkyl" refers to an alkoxy group, as defined herein, appended to an alkyl

group, as defined herein. Exemplary alkoxyalkyl groups include methoxymethyl, methoxyethyl, isopropoxymethyl, and the like.

"Alkoxyhaloalkyl" refers to an alkoxy group, as defined herein, appended to a haloalkyl group, as defined herein. Exemplary alkoxyhaloalkyl groups include 4-methoxy-2-chlorobutyl and the like.

"Cycloalkoxy" refers to $R_{54}O$-, wherein R_{54} is a cycloalkyl group or a bridged cycloalkyl group, as defined herein. Exemplary cycloalkoxy groups include cyclopropyloxy, cyclopentyloxy, cyclohexyloxy, and the like.

"Cycloalkylthio" refers to $R_{54}S$-, wherein R_{54} is a cycloalkyl group or a bridged cycloalkyl group, as defined herein. Exemplary cycloalkylthio groups include cyclopropylthio, cyclopentylthio, cyclohexylthio, and the like.

"Haloalkoxy" refers to an alkoxy group, as defined herein, in which one or more of the hydrogen atoms on the alkoxy group are substituted with halogens, as defined herein. Exemplary haloalkoxy groups include 1,1,1-trichloroethoxy, 2-bromobutoxy, and the like.

"Hydroxy" refers to -OH.

"Oxo " refers to =O.

"Oxy " refers to $-O^- R_{77}^+$ wherein R_{77} is an organic or inorganic cation.

"Organic cation" refers to a positively charged organic ion. Exemplary organic cations include alkyl substituted ammonium cations, and the like.

"Inorganic cation" refers to a positively charged metal ion. Exemplary inorganic cations include Group I metal cations such as for example, sodium, potassium, and the like.

"Hydroxyalkyl" refers to a hydroxy group, as defined herein, appended to an alkyl group, as defined herein.

"Nitrate" refers to $-O-NO_2$.

"Nitrite" refers to -O-NO.

"Thionitrate" refers to $-S-NO_2$.

"Thionitrite" and "nitrosothiol" refer to -S-NO.

"Nitro" refers to the group $-NO_2$ and "nitrosated" refers to compounds that have been substituted therewith.

"Nitroso" refers to the group -NO and "nitrosylated" refers to compounds that have been substituted therewith.

"Nitrile" and "cyano" refer to -CN.

"Halogen" or "halo" refers to iodine (I), bromine (Br), chlorine (Cl), and/or fluorine

(F).

"Amino " refers to -NH_2, an alkylamino group, a dialkylamino group, an arylamino group, a diarylamino group, an alkylarylamino group or a heterocyclic ring, as defined herein.

"Alkylamino" refers to $R_{50}NH$-, wherein R_{50} is an alkyl group, as defined herein. Exemplary alkylamino groups include methylamino, ethylamino, butylamino, cyclohexylamino, and the like.

"Arylamino" refers to $R_{55}NH$-, wherein R_{55} is an aryl group, as defined herein.

"Dialkylamino" refers to $R_{52}R_{53}N$-, wherein R_{52} and R_{53} are each independently an alkyl group, as defined herein. Exemplary dialkylamino groups include dimethylamino, diethylamino, methyl propargylamino, and the like.

"Diarylamino" refers to $R_{55}R_{60}N$-, wherein R_{55} and R_{60} are each independently an aryl group, as defined herein.

"Alkylarylamino or arylalkylamino" refers to $R_{52}R_{55}N$-, wherein R_{52} is an alkyl group, as defined herein, and R_{55} is an aryl group, as defined herein.

"Alkylarylalkylamino " refers to $R_{52}R_{79}N$-, wherein R_{52} is an alkyl group, as defined herein, and R_{79} is an arylalkyl group, as defined herein.

"Alkylcycloalkylamino " refers to $R_{52}R_{80}N$-, wherein R_{52} is an alkyl group, as defined herein, and R_{80} is an cycloalkyl group, as defined herein.

"Aminoalkyl " refers to an amino group, an alkylamino group, a dialkylamino group, an arylamino group, a diarylamino group, an alkylarylamino group or a heterocyclic ring, as defined herein, to which is appended an alkyl group, as defined herein. Exemplary aminoalkyl groups include dimethylaminopropyl, diphenylaminocyclopentyl, methylaminomethyl, and the like.

"Aminoaryl " refers to an aryl group to which is appended an alkylamino group, a arylamino group or an arylalkylamino group. Exemplary aminoaryl groups include anilino, N-methylanilino, N-benzylanilino, and the like.

"Thio" refers to –S-.

"Sulfinyl" refers to -S(O)-.

"Methanthial" refers to -C(S)-.

"Thial" refers to =S.

"Sulfonyl" refers to -$S(O)_2$-.

"Sulfonic acid" refers to -$S(O)_2OR_{76}$, wherein R_{76} is a hydrogen, an organic cation or

an inorganic cation, as defined herein.

"Alkylsulfonic acid" refers to a sulfonic acid group, as defined herein, appended to an alkyl group, as defined herein.

"Arylsulfonic acid" refers to a sulfonic acid group, as defined herein, appended to an aryl group, as defined herein

"Sulfonic ester" refers to $-S(O)_2OR_{58}$, wherein R_{58} is an alkyl group, an aryl group, or an aryl heterocyclic ring, as defined herein.

"Sulfonamido" refers to $-S(O)_2-N(R_{51})(R_{57})$, wherein R_{51} and R_{57} are each independently a hydrogen atom, an alkyl group, an aryl group or an arylheterocyclic ring, as defined herein, or R_{51} and R_{57} taken together with the nitrogen to which they are attached are a heterocyclic ring or a bridged cycloalkyl group, as defined herein.

"Alkylsulfonamido" refers to a sulfonamido group, as defined herein, appended to an alkyl group, as defined herein.

"Arylsulfonamido" refers to a sulfonamido group, as defined herein, appended to an aryl group, as defined herein.

"Alkylthio" refers to $R_{50}S-$, wherein R_{50} is an alkyl group, as defined herein (preferably a lower alkyl group, as defined herein).

"Arylthio" refers to $R_{55}S-$, wherein R_{55} is an aryl group, as defined herein.

"Arylalkylthio" refers to an aryl group, as defined herein, appended to an alkylthio group, as defined herein.

"Alkylsulfinyl" refers to $R_{50}-S(O)-$, wherein R_{50} is an alkyl group, as defined herein.

"Alkylsulfonyl" refers to $R_{50}-S(O)_2-$, wherein R_{50} is an alkyl group, as defined herein.

"Alkylsulfonyloxy" refers to $R_{50}-S(O)_2-O-$, wherein R_{50} is an alkyl group, as defined herein.

"Arylsulfinyl" refers to $R_{55}-S(O)-$, wherein R_{55} is an aryl group, as defined herein.

"Arylsulfonyl" refers to $R_{55}-S(O)_2-$, wherein R_{55} is an aryl group, as defined herein.

"Arylsulfonyloxy" refers to $R_{55}-S(O)_2-O-$, wherein R_{55} is an aryl group, as defined herein.

"Amidyl" refers to $R_{51}C(O)N(R_{57})-$ wherein R_{51} and R_{57} are each independently a hydrogen atom, an alkyl group, an aryl group or an arylheterocyclic ring, as defined herein.

"Ester" refers to $R_{51}C(O)O-$ wherein R_{51} is a hydrogen atom, an alkyl group, an aryl group or an arylheterocyclic ring, as defined herein.

"Carbamoyl" refers to -O-C(O)N(R_{51})(R_{57}), wherein R_{51} and R_{57} are each independently a hydrogen atom, an alkyl group, an aryl group or an arylheterocyclic ring, as defined herein, or R_{51} and R_{57} taken together with the nitrogen to which they are attached are a heterocyclic ring or a bridged cycloalkyl group, as defined herein.

"Carboxyl" refers to –C(O)OR_{76}, wherein R_{76} is a hydrogen, an organic cation or an inorganic cation, as defined herein.

"Carbonyl" refers to –C(O)-.

"Alkylcarbonyl" refers to R_{52}-C(O)-, wherein R_{52} is an alkyl group, as defined herein.

"Arylcarbonyl" refers to R_{55}-C(O)-, wherein R_{55} is an aryl group, as defined herein.

"Arylalkylcarbonyl" refers to R_{55}-R_{52}-C(O)-, wherein R_{55} is an aryl group, as defined herein, and R_{52} is an alkyl group, as defined herein.

"Alkylarylcarbonyl" refers to R_{52}-R_{55}-C(O)-, wherein R_{55} is an aryl group, as defined herein, and R_{52} is an alkyl group, as defined herein.

"Heterocyclicalkylcarbonyl" refer to R_{78}C(O)- wherein R_{78} is a heterocyclicalkyl group, as defined herein.

"Carboxylic ester" refers to -C(O)OR_{58}, wherein R_{58} is an alkyl group, an aryl group or an aryl heterocyclic ring, as defined herein.

"Alkylcarboxylic acid" and "alkylcarboxyl" refer to an alkyl group, as defined herein, appended to a carboxyl group, as defined herein.

"Alkylcarboxylic ester" refers to an alkyl group, as defined herein, appended to a carboxylic ester group, as defined herein.

"Arylcarboxylic acid" refers to an aryl group, as defined herein, appended to a carboxyl group, as defined herein.

"Arylcarboxylic ester" and "arylcarboxyl" refer to an aryl group, as defined herein, appended to a carboxylic ester group, as defined herein.

"Carboxamido" refers to -C(O)N(R_{51})(R_{57}), wherein R_{51} and R_{57} are each independently a hydrogen atom, an alkyl group, an aryl group or an arylheterocyclic ring, as defined herein, or R_{51} and R_{57} taken together with the nitrogen to which they are attached are a heterocyclic ring or a bridged cycloalkyl group, as defined herein.

"Alkylcarboxamido" refers to an alkyl group, as defined herein, appended to a carboxamido group, as defined herein.

"Arylcarboxamido" refers to an aryl group, as defined herein, appended to a carboxamido group, as defined herein.

"Oxime" refers to -C(=N-OR$_{81}$) wherein R$_{81}$is a hydrogen, an alkyl group, an aryl group, an alkylsulfonyl group, an arylsulfonyl group, a carboxylic ester, an alkylcarbonyl group, an arylcarbonyl group, a carboxamido group, an alkoxyalkyl group or an alkoxyaryl group.

"Urea" refers to -N(R$_{59}$)-C(O)N(R$_{51}$)(R$_{57}$) wherein R$_{51}$, R$_{57}$, and R$_{59}$ are each independently a hydrogen atom, an alkyl group, an aryl group or an arylheterocyclic ring, as defined herein, or R$_{51}$ and R$_{57}$ taken together with the nitrogen to which they are attached are a heterocyclic ring or a bridged cycloalkyl group, as defined herein.

"Phosphoryl" refers to -P(R$_{70}$)(R$_{71}$)(R$_{72}$), wherein R$_{70}$ is a lone pair of electrons, sulfur or oxygen, and R$_{71}$ and R$_{72}$ are each independently a covalent bond, a hydrogen, a lower alkyl, an alkoxy, an alkylamino, a hydroxy or an aryl, as defined herein.

"Silyl" refers to -Si(R$_{73}$)(R$_{74}$)(R$_{75}$), wherein R$_{73}$, R$_{74}$ and R$_{75}$ are each independently a covalent bond, a lower alkyl, an alkoxy, an aryl or an arylalkoxy, as defined herein.

Two broad classes of cardiovascular diseases or disorders are more prevalent among blacks than whites and serve as areas in need of investigative efforts. Hypertension and left ventricular hypertrophy, two related yet independent risk factors for coronary heart disease, are significantly more prevalent among blacks than whites. Blacks also have higher rates of angiographically normal coronary arteries despite a higher prevalence of risk factors for coronary atherosclerosis, and greater morbidity and mortality from coronary heart disease than whites. These paradoxical observations have led some investigators to postulate that blacks harbor a diathesis of the microvasculature that limits perfusion and serves as a stimulus for vascular smooth muscle cell and cardiomyocyte hypertrophy, which, in turn, leads to hypertension and left ventricular hypertrophy, respectively. The underlying basis for this vascular diathesis may involve the endothelium, which has a limited capacity to generate vasodilator and antiproliferative factors or an increased capacity to produce vasoconstrictor and proliferative factors; the vascular smooth muscle cell, which manifests increased sensitivity to vasoconstrictor and proliferative factors; or both, in these individuals.

A major product of the normal blood vessel that may play a role in the vascular diathesis of blacks is endothelium-derived nitric oxide (NO). Nitric oxide produced by the endothelial cells induces vascular smooth muscle cell relaxation, contributing importantly

to resting vascular tone. In addition, NO inhibits vascular smooth muscle cell proliferation and induces apoptosis in smooth muscle cells, which leads to the release of basic fibroblast growth factor and vascular endothelial cell growth factor, in turn supporting endothelial cell proliferation. This sequence of cellular responses is believed to sustain angiogenesis under hypoxic or ischemic conditions.

The role of nitric oxide in the vascular diathesis of blacks is illustrated by the consequences of nitric oxide insufficiency in the normal responses of the vasculature to nitric oxide. Nitric oxide insufficiency suppresses renin release from the juxtaglomerular cells, and induces a sodium chloride/volume sensitive increase in blood pressure. Furthermore, nitric oxide insufficiency leads to an increased sensitivity of vascular smooth muscle cells to vasoconstrictors, such as angiotensin II and catecholamines, which amplify the increase in vascular resistance.

Nitric oxide insufficiency promotes vascular smooth muscle cell proliferation following vascular injury, and sustains smooth muscle cell and cardiomyocyte hypertrophy in response to catecholamines and angiotensin II. Furthermore, inadequate nitric oxide leads to increased production of extracellular matrix with consequent myocardial fibrosis.

These many cardiovascular responses that result from inadequate NO in the vasculature have clear clinical correlates in the black population. The clinical vascular phenotype of blacks that distinguishes them from whites with similar cardiovascular diseases or disorders is one of salt-sensitive, low-renin hypertension; left ventricular hypertrophy disproportionate to after load and with an inadequate angiogenic response; and microvascular ischemia in the absence of significant epicardial coronary artery disease. The net pathophysiological consequences of these effects are increased peripheral vascular resistance with accompanying arterial hypertension; and an inadequately vascularized, fibrotic increase in left ventricular mass with accompanying diastolic dysfunction and microvascular ischemia.

Nitric oxide insufficiency states can be a consequence of reduced synthesis of nitric oxide, enhanced inactivation of nitric oxide, or both. Possible candidate mechanisms include alterations in the genes that code for endothelial nitric oxide synthase or the inducible microvascular and cardiomyocyte nitric oxide synthase leading to reduced expression of a normal gene product or appropriate expression of a less active gene product; reduction in the enzymatic activity of nitric oxide synthase owing to inadequate cofactor concentrations; or enhanced inactivation of nitric oxide by oxidant stress.

Data obtained by the inventors in cultured cells, animal models, and human patients suggest that increased oxidant stress is central to the vascular diathesis of and consequent cardiovascular diseases or disorders common among African Americans. Possible candidate mechanisms for the oxidant stress include enhanced production of reactive oxygen species (ROS), decreased antioxidant defenses, or both. The inventors make no *a priori* assumptions about the temporal or causative relationship between oxidant stress and the vascular phenotype of blacks: oxidant stress may both precede the development of the vascular diathesis and promote its progression once established. Recent data suggest that enhanced ROS production accompanies essential hypertension, atherosclerosis, thrombosis, and diabetes mellitus, and appears in each case, at the very least, to be important in the progression of established disease, if not in its actual genesis.

Endothelium-derived relaxing factor (EDRF), first described by Furchgott et al, *Nature*, 299:373-376 (1980), is an important mediator of vascular function. This endothelial product activates guanylyl cyclase in vascular smooth muscle cells and platelets, leading to vasorelaxation and platelet inhibition, respectively (Loscalzo et al, *Prog Cardiovasc Dis*, 38:87-104 (1995)). The chemical nature of EDRF has been studied using a variety of pharmacological and analytical techniques, and is NO (Ignarro et al, *Circ Res*, 61:866-879 (1987); Palmer et al, *Nature*, 327:524-526 (1987)).

Nitric oxide is synthesized by one of several isoforms of the NO synthase (NOS) family of enzymes, two of which are found in the vasculature, endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is synthesized by endothelial cells, while iNOS is synthesized by a variety of cell types, including vascular smooth muscle cells, fibroblasts, and (principally microvascular) endothelial cells (Balligand et al, *Am J Physiol*, 268:H1293-1303 (1995)). These enzymes produce NO as a result of the five-electron oxidation of L-arginine to L-citrulline; requisite cofactors include calcium-calmodulin, O_2, FAD, FMN, tetrahydrobiopterin thiols, heme, and NADPH. (Moncada et al, *N EngI J Med*, 329:2002-2012 (1993)).

The role of NO in the cardiovascular system has become increasingly apparent over the past fifteen years (Loscalzo et al, *Prog Cardiovasc Dis*, 38:87-104 (1995)). Nitric oxide contributes importantly to resting tone in conductance as well as resistance arteries (Ouyyumi et al, *J Clin Invest*, 95:1747-1755 (1995)), and plays a critical role in the maintenance of peripheral vascular resistance and arterial pressure responses. Inhibition of NOS activity is associated with enhanced vascular sensitivity to vasoconstrictors, such as

norepinephrine and angiotensin II (Conrad et al, *Am J Physiol*, 262:R1137-R1144 (1992)), and this effect appears to be mediated, in part, by increased calcium sensitivity (Bank et al, *Hypertension*, 24:322-328 (1994)). Nitric oxide release from the cardiovascular regulatory center in the brain may also be involved in the central regulation of blood pressure, suggesting a role for neuronal NOS in the regulation of vascular tone (Cabrera et al, *Biochem Biophys Res Comm*, 206:77-81 (1995); Mattson et al, *Hypertension*, 28:297-303 (1996)).

Nitric oxide activates renin gene expression in the kidney, and is involved in the baroreceptor-mediated regulation of renin gene expression (Schricker et al, *Pflug Arch*, 428:261-268 (1994)). The dependence of blood pressure on salt intake appears to depend on NO, and NO deficiency states are associated with salt-sensitivity (Tolins et al, *Kidney Internat*, 46:230-236 (1994)). Selective inhibition of iNOS in Dahl R rats has been shown to lead to salt-sensitivity and to the development of salt-dependent hypertension similar to Dahl S rats (Rudd et al, *Am J Physiol*, 277: H732-H739 (1999)). In addition, mice deficient in iNOS (iNOS gene eliminated by targeted disruption) may develop hypertension in response to salt feeding (Rudd et al, *Circulation*, 98:1A (1998)).

Nitric oxide also affects myocardial contractility, and does so both by mediating muscarinic-cholinergic slowing of the heart rate and the contractile response to beta-adrenergic stimulation (Balligand et al, *Proc Nat'l Acad Sci USA*, 90:347-351 (1993)). This latter effect appears to be mediated *in vivo* through the vagus nerve (Hare et al, *J Clin Invest*, 95:360-366 (1995)).

In both vascular smooth muscle cells and cardiomyocytes, NO inhibits cellular proliferation and limits the proliferative response to growth-promoting substances (Garg et al, *J Clin Invest*, 83:1774-1777 (1986)). Left ventricular hypertrophy tends to occur in adult hearts with inadequate capillary proliferation, and this may account for the microvascular ischemia noted in patients with hypertrophy. Capillary proliferation is generally held to be a rare event in normal adult mammalian hearts. However, recent data from a hypertensive rat model, in which left ventricular hypertrophy commonly occurs, show that treatment with a low-dose of an angiotensin-converting enzyme inhibitor insufficient to prevent hypertension and left ventricular hypertrophy can, nonetheless, evoke capillary angiogenesis. Compared with untreated controls, treatment with the angiotensin converting enzyme inhibitor increased myocardial capillary proliferation (Unger et al, *Hypertension*, 20:478482 (1992)), and this effect was believed to be a consequence of inhibiting the

degradation and potentiating the action of bradykinin. Bradykinin increases myocardial blood flow by inducing release of NO from microvascular endothelial cells, and increased blood flow is a powerful stimulus for capillary proliferation (Mall et al, *Bas Res Cardiol*, 85:531-540 (1990)).

Normal metabolic processes in vascular cells are associated with the generation of reactive oxygen intermediates that must be neutralized to limit oxidative damage and cellular dysfunction. In the setting of common cardiovascular diseases or disorders or in the presence of common risk factors for atherothrombotic disease, reactive oxygen species (ROS) are generated in abundance, and their rate of synthesis and flux typically exceeds the capacity of endogenous antioxidant mechanisms. Hypercholesterolemia, hyperglycemia (Keaney et al, *Circulation*, 99:189-191 (1999)), cigarette smoking, hyperhomocysteinemia, hypertension, and frank atherosclerosis are all accompanied by an increase in plasma and tissue ROS generation. Superoxide anion, hydrogen peroxide, hydroxyl radical, peroxynitrite, and lipid peroxides all increase in these settings. What remains unknown is whether or not the increase in ROS in these disorder is a primary event, a secondary consequence of the underlying process, or both.

Endogenous antioxidants important for the neutralization (i.e., reduction) of ROS can be categorized into two groups: small-molecule antioxidants and antioxidant enzymes. The former group comprises molecules such as GSH, NADPH, α-tocopherol, vitamin C, and ubiquinol-10; while the latter group comprises the superoxide dismutases, catalase, and glutathione peroxidases. Deficiencies in several of these molecular species have been shown to lead to increased steady-state levels of ROS and vascular dysfunction, including increased platelet activation, arterial thrombosis (Freedman et al, *J Clin Invest*, 97:979-987 (1996); Freedman et al, *Circulation*, 98:1481-1486 (1998)), and reduced production of platelet-derived NO (Kenet et al, *Arterio Thromb Vasc Biol*, 19(8): 2017-2023 (1999)), which is important for limiting expansion of a platelet thrombus (Freedman et al, *Circ Res*, 84:1416-142 (1999)).

ROS generation accompanies the vascular dysfunction associated with several models of atherothrombotic and hypertensive vascular diseases. Hyperhomo-cysteinemic mice (i.e., cystathionine ß-synthase knock-out mice) (Eberhardt et al, *Circulation*, 98:144 (1998)), cellular glutathione peroxidase-deficient mice (i.e., cellular glutathione peroxidase knock-out mice), and salt-induced hypertensive rats (i.e., salt-fed Dahl S rats) (Trolliet et al, *Circulation*, 98:1-725 (1998)) all manifest increased vascular ROS, and this increase in

ROS is accompanied by reduced NO bioactivity through oxidative inactivation. Endothelial function and NO availability can be improved by improving antioxidant status with a cysteine precursor (Vita et al, *J Clin Invest*, 101:1408-1414 (1998)). In addition, α-tocopherol leads to platelet inhibition (Freedman et al, *Circulation*, 94:2434-2440 (1996)) as one mechanism of its atherothrombotic benefit (Stephens et al, Lancet, 347:781-786 (1996)). Salt-loading salt-sensitive individuals (Dahl S rats) lead to an approximate 5-fold increase in plasma F_2-isoprostanes (8-epi-prostaglandin F_2), and this increase precedes the development of florid hypertension. These data all support the role of oxidant stress in the genesis or evolution of vascular dysfunction and disease, and the importance of antioxidant mechanisms in preventing this pathobiology, particularly with regard to African Americans.

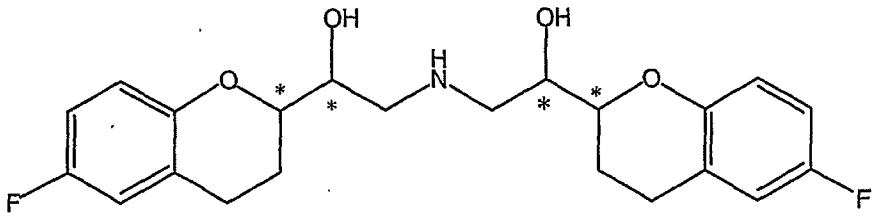
In support of the mechanisms illustrated above, minimum forearm vascular resistance is significantly higher among normotensive blacks than whites (Bassett et al, *Am J Hypertension*, 5:781-786 (1992)), and forearm blood-flow responses to isoproterenol are markedly attenuated in normotensive blacks, suggesting a blunted $ß_2$-vasodilator response in these individuals (Lang et al, *N EngI J Med*, 333:155-160 (1995)). Blacks tend to have greater left ventricular mass than whites for any given level of blood pressure (Koren et al, *Am J Hypertension*, 6:815-823 (1993); Chaturvedi et al, *J Am Coll Cardiol*, 24:1499-1505 (1994)). While not quantitated in any necropsy study, this response is likely to be accompanied by inadequate capillary angiogenesis, which, in turn, may account for the diastolic dysfunction and the microvascular ischemia observed in blacks. Interestingly, blacks have been observed to have low levels of urinary kallikrein (Zinner et al, *Am J Epidemiol*, 104:124-132 (1976); Levy et al, *J Clin Invest*, 60:129-138 (1977)), the enzyme responsible for the generation of bradykinin from high-molecular-weight kininogen. Thus, were a similar abnormality in bradykinin and bradykinin-mediated NO production to exist in the coronary vasculature, attenuated blood flow responses may result that would limit capillary angiogenic responses and prevent the endothelial proliferative effects of locally derived NO.

As discovered and described herein, African Americans have a unique vascular diathesis that may serve as the basis for clinically important cardiovascular syndromes. For example, differences in the outcome of left ventricular dysfunction may be a consequence of the enhanced (perhaps salt-dependent) increase in oxidant stress coupled with microvascular endothelial dysfunction and an inadequately vascularized, hypertrophied left ventricle. This constellation of pathophysiological abnormalities may provide the substrate

for the important differences in outcome between blacks and whites with left ventricular dysfunction (Dreis et al, *N Engl J Med*, 340:609-616 (1999)). In addition, these observations and their clinical consequences suggest that blacks with abnormal endothelial function and nitric oxide insufficiency states would derive direct and, perhaps, disproportionate clinical benefit from enhancing nitric oxide in the vasculature, either by improving endothelial function, providing exogenous nitric oxide donors, or both.

The invention is directed to the treatment and/or prevention of vascular diseases characterized by nitric oxide insufficiency; and for treating and/or preventing Raynaud's syndrome and for treating and/or preventing cardiovascular diseases or disorders by administering nebivolol that is optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, that is optionally substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated). Preferably, the nitrosated and/or nitrosylated nebivolol, and/or its nitrosylated and/or nitrosated metabolites are administered as a pharmaceutical composition that further comprises a pharmaceutically acceptable carrier or diluent. The novel compounds and novel compositions of the invention are described in more detail herein.

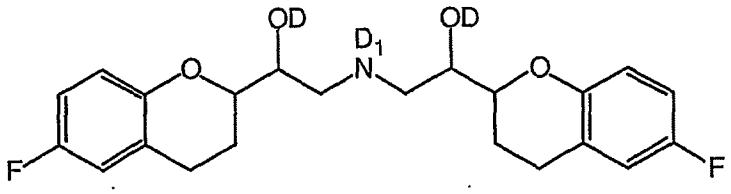
Nebivolol ((±)-(RSSS)-αα'-(iminobis(methylene)bis-(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol) is a long lasting cardioselective ß-blocker having mild vasodilating properties. It is administered as its hydrochloride salt as mixture of equal amounts of its 2 enantiomers (SRRR and RSSS) under the tradenames NEBILET®, NEBILOX® or LOBIVON®. The structure of nebivolol with its four stereogenic centers indicated with an asterisk is shown below:



The absorption of nebivolol is rapid and it is extensively metabolized, partly to active metabolites. Compounds contemplated for use in the invention include nebivolol and all its metabolites known in the art and include those described herein, such as, for example,

the hydroxy derivatives of nebivolol, the N-alkylated metabolites of nebivolol, and the like. Nebivolol and its metabolites are disclosed in, for example, U. S. Patent Nos. 4,654,362, 5,759,580, 6,075,046, and in EP 0 145 067, EP 0 334 429, and in WO 95/22325 and WO 96/19987; Van Lommen et al., *J. Pharm. Belg.*, 45(6): 355-360 (1990); Chandrasekhar, S. et al., *Tetrahedron*, 56(34): 6339-6344 (2000); and Fendrickx et at., *J. Chromatogr. A.*, 729: 341-354 (1996); the disclosures of each of which are incorporated by reference herein in their entirety.

In one embodiment, the invention describes nitrosated and/or nitrosylated nebivolol of Formula (I), isomers thereof, and pharmaceutically acceptable salts thereof;



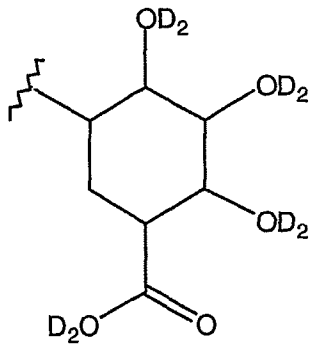
I

wherein:

D is hydrogen, Q, K or R_5;

D_1 is hydrogen or R_5;

R_5 is:



D_2 is hydrogen, Q or K;

Q is –NO or -NO_2;

K is $-W_a-E_b-(C(R_e)(R_f))_p-E_c-(C(R_e)(R_f))_x-W_d-(C(R_e)(R_f))_y-W_i-E_j-W_g-(C(R_e)(R_f))_z-T-Q$;

a, b, c, d, g, i and j are each independently an integer from 0 to 3;

p, x, y and z are each independently an integer from 0 to 10;

W at each occurrence is independently -C(O)-, -C(S)-, -T-, $-(C(R_e)(R_f))_h-$, an alkyl group, an aryl group, a heterocyclic ring, an arylheterocyclic ring, or $-(CH_2CH_2O)_q-$;

E at each occurrence is independently -T-, an alkyl group, an aryl group, $-(C(R_e)(R_f))_h-$, a heterocyclic ring, an arylheterocyclic ring, or $-(CH_2CH_2O)_q-$;

h is an integer form 1 to 10;

q is an integer from 1 to 5;

R_e and R_f are each independently a hydrogen, an alkyl, a cycloalkoxy, a halogen, a hydroxy, an hydroxyalkyl, an alkoxyalkyl, an arylheterocyclic ring, an alkylaryl, an alkylcycloalkyl, an alkylheterocyclic ring, a cycloalkylalkyl, a cycloalkylthio, a cycloalkenyl, an heterocyclicalkyl, an alkoxy, a haloalkoxy, an amino, an alkylamino, a dialkylamino, an arylamino, a diarylamino, an alkylarylamino, an alkoxyhaloalkyl, a haloalkoxy, a sulfonic acid, a sulfonic ester, an alkylsulfonic acid, an arylsulfonic acid, an arylalkoxy, an alkylthio, an arylthio, a cyano an aminoalkyl, an aminoaryl, an aryl, an arylalkyl, an alkylaryl, a carboxamido, a alkylcarboxamido, an arylcarboxamido, an amidyl, a carboxyl, a carbamoyl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarbonyl, an arylcarbonyl, an ester, a carboxylic ester, an alkylcarboxylic ester, an arylcarboxylic ester, a haloalkoxy, a sulfonamido, an alkylsulfonamido, an arylsulfonamido, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfonyl, arylsulphonyloxy, a sulfonic ester, a urea, a phosphoryl, a nitro, W_h, -T-Q , or $-(C(R_e)(R_f))_k-T-Q$, or R_e and R_f taken together with the carbons to which they are attached form a carbonyl, a methanthial, a heterocyclic ring, a cycloalkyl group, an aryl group, an oxime or a bridged cycloalkyl group;

k is an integer from 1 to 3;

T at each occurrence is independently a covalent bond, a carbonyl, an oxygen, $-S(O)_o-$ or $-N(R_a)R_i-$;

o is an integer from 0 to 2;

R_a is a lone pair of electrons, a hydrogen or an alkyl group;

R_i is a hydrogen, an alkyl, an aryl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarboxylic ester, an arylcarboxylic ester, an alkylcarboxamido, an arylcarboxamido, an alkylaryl, an alkylsulfinyl, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfinyl, an

arylsulfonyl, arylsulphonyloxy, a sulfonamido, a carboxamido, a carboxylic ester, an aminoalkyl, an aminoaryl, $-CH_2-C(T-Q)(R_e)(R_f)$, a bond to an adjacent atom creating a double bond to that atom, $-(N_2O_2-)^-\bullet M^+$, wherein M^+ is an organic or inorganic cation;

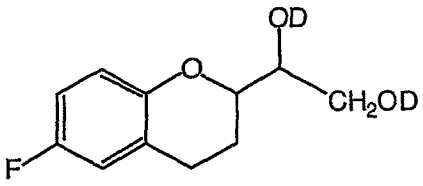
with the proviso that the compound of Formula (I) must contain at least one nitrite, nitrate, thionitrite or thionitrate group.

In cases where R_e and R_f are a heterocyclic ring or R_e and R_f taken together with the hetero atom to which they are attached are a heterocyclic ring, then R_i can be a substituent on any disubstituted nitrogen contained within the radical where R_i is as defined herein.

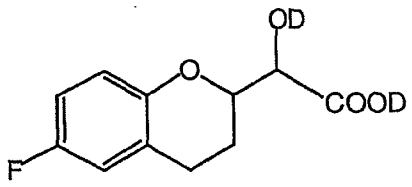
In cases where multiple designations of variables that reside in sequence are chosen as a "covalent bond" or the integer chosen is 0, the intent is to denote a single covalent bond connecting one radical to another. For example, E_0 would denote a covalent bond, while E_2 denotes (E-E) and $(C(R_e)(R_f))_2$ denotes $-C(R_e)(R_f)-C(R_e)(R_f)-$, where R_e and R_f at each occurrence are each independently selected from those moieties defined herein.

Another embodiment of the invention describes the nitrosated and/or nitrosylated metabolites of nebivolol of Formula (II), Formula (III), Formula (IV) or Formula (V), isomers thereof, and pharmaceutically acceptable salts thereof;

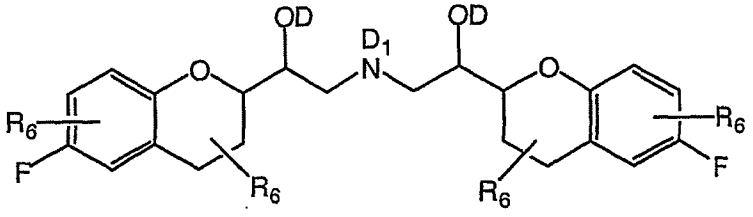
wherein the compounds of Formula (II), Formula (III), Formula (IV) and Formula (V) are:



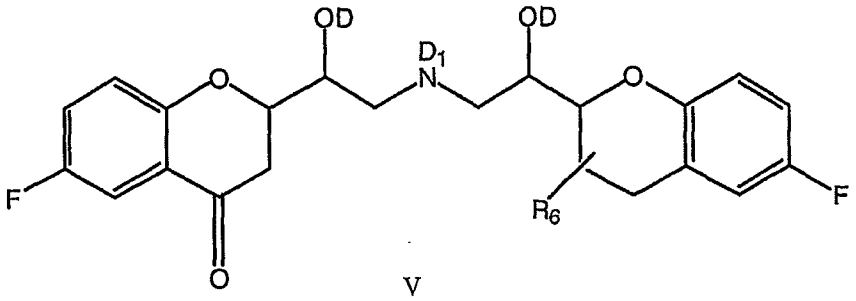
II



III



IV



V

wherein:

R_6 at each occurrence is independently a hydrogen, a hydroxy or -OD;

D and D_1 are as defined herein; and

with the proviso that the compounds of Formula (II), Formula (III), Formula (IV) and Formula (V), must contain at least one nitrite, nitrate, thionitrite or thionitrate group.

Compounds of the invention, that have one or more asymmetric carbon atoms, can exist as the optically pure enantiomers, pure diastereomers, mixtures of enantiomers, mixtures of diastereomers, racemic mixtures of enantiomers, diastereomeric racemates or mixtures of diastereomeric racemates. It is to be understood that the invention anticipates and includes within its scope all such isomers and mixtures thereof.

The parent nebivolol compound and its metabolites can be synthesized by one skilled in the art following the methods described in, for example, U. S. Patent Nos. 4,654,362, 5,759,580, 6,075,046, and in EP 0 145 067, EP 0 334 429, and in WO 95/22325 and WO 96/19987; Van Lommen et al., *J. Pharm. Belg.*, 45(6): 355-360 (1990); Chandrasekhar, S. et al., *Tetrahedron*, 56(34): 6339-6344 (2000); and Fendrickx et at., *J. Chromatogr. A.*, 729: 341-354 (1996); the disclosure of each of which are incorporated by reference herein in their entirety. The parent nebivolol compound and its metabolites can be nitrosated and/or nitrosylated through one or more sites such as oxygen (hydroxyl condensation), sulfur (sulfhydryl condensation), and/or nitrogen. The nitrosated and nitrosylated compounds of the invention can be prepared using conventional methods known to one skilled in the art. For example, known methods for nitrosylating compounds are described in U.S. Patent Nos. 5,380,758 and 5,703,073; WO 97/27749; WO 98/19672; and Oae et al, *Org. Prep. Proc. Int.*, *15*(3):165-198 (1983), the disclosures of each of which

are incorporated by reference herein in their entirety.

Compounds of the invention can be synthesized following the methods described herein. The reactions are performed in solvents appropriate to the reagents, and materials used are suitable for the transformations being effected. It is understood by one skilled in the art of organic synthesis that the functionality present in the molecule must be consistent with the chemical transformation proposed. This will, on occasion, necessitate judgment by the routineer as to the order of synthetic steps, protecting groups required, and deprotection conditions. Substituents on the starting materials may be incompatible with some of the reaction conditions required in some of the methods described, but alternative methods and substituents compatible with the reaction conditions will be readily apparent to one skilled in the art. The use of sulfur and oxygen protecting groups is known in the art for protecting thiol and alcohol groups against undesirable reactions during a synthetic procedure and many such protecting groups are known, e.g., T.H. Greene and P.G.M. Wuts, *Protective Groups in Organic Synthesis*, John Wiley & Sons, New York (1999), which is incorporated herein in its entirety.

Compounds of the invention can be synthesized as shown in **Figures 1** to **16**. Nitroso compounds of Formula (I) wherein R_e, R_f, and p are defined as herein, D^1 is hydrogen, $P^{1'}$ is an acetyl or trifluoroacetyl ester, and hydrogen and an O-nitrosylated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 1**. The amine group of Formula **1** is protected to afford the compound of Formula **2**, wherein P^3 is as defined herein. Preferred protecting groups for the amine are as a carbamate, such as, a benzyl or tert-butyl carbamate, or an amide, such as, a trifluoroacetamide. An alcohol group of Formula **2** is converted to the ester of Formula **3**, wherein p, R_e and R_f are defined herein, by reaction with an appropriate protected alcohol containing activated acylating agent, wherein P^1 is as defined herein. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected alcohol containing acid or condensing the alcohol and protected alcohol containing acid in the presence of a dehydrating agent, such as, dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC·HCl) with or without a catalyst, such as, 4-dimethyaminopyridine (DMAP) or 1-hydroxybenzotriazole (HOBt). Preferred protecting groups for the alcohol moiety are silyl ethers, such as, a trimethylsilyl or a tert-butyldimethylsilyl ether. Protection of the remaining secondary alcohol as an ester, such as, an acetyl or trifluoroacetyl ester, followed by deprotection of

the silylated hydroxyl moiety (fluoride ion is the preferred method for removing silyl ether protecting groups) and then reaction with a suitable nitrosylating agent, such as, thionyl chloride nitrite, thionyl dinitrite, or nitrosonium tetrafluoroborate, in a suitable anhydrous solvent, such as, dichloromethane, THF, DMF, or acetonitrile, with or without an amine base, such as, pyridine or triethylamine, affords the compound of Formula **4**. The compound of Formula **4** is then converted to the compound of Formula **IA** by deprotecting the amine and remaining hydroxyl group. Hydrogen in the presence of a transition metal catalyst, such as, palladium or platinum, is a preferred method for removing benzyl ether and benzyl carbamate protecting groups, strong anhydrous acids, such as, trifluoroacetic acid or hydrochloric acid in methanol, dioxane or ethyl acetate are preferred for removing the t-butyl carbamate protecting group and mild base, such as, aqueous sodium or potassium carbonate or ammonia in methanol, are the preferred methods for removing trifluoroacetamide, trifluoroacetyl ester or acetyl ester protecting groups.

Nitroso compounds of Formula (I) wherein P^3, R_e, R_f, and p are as defined herein, D^1 is hydrogen, and a S-nitrosylated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 2**. The compound of Formula **2**, wherein P^3 is as defined herein, with the preferred protecting group for the amine being a carbamate, such as, a t-butyl carbamate, is converted to the ester of Formula **5**, wherein p, R_e and R_f, are as defined herein, by reaction with an appropriate protected thiol containing activated acylating agent, wherein P^2 is as defined herein. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected thiol containing acid or condensing the alcohol and protected thiol containing acid in the presence of a dehydrating agent, such as, DCC or EDAC·HCl, with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the thiol moiety are as a thioester, such as, a thioacetate or thiobenzoate, as a disulfide, as a thiocarbamate, such as, N-methoxymethyl thiocarbamate, or as a thioether, such as, a paramethoxybenzyl thioether, a tetrahydropyranyl thioether or a 2,4,6-trimethoxybenzyl thioether. Deprotection of the thiol moiety (zinc in dilute aqueous acid, triphenylphosphine in water and sodium borohydride are the preferred methods for reducing disulfide groups, while aqueous base is typically utilized to hydrolyze thioesters, and N-methoxymethyl thiocarbamates and mercuric trifluoroacetate, or silver nitrate are the preferred methods to remove a paramethoxybenzyl thioether, a tetrahydropyranyl thioether or a 2,4,6-trimethoxybenzyl thioether group) affords a compound of Formula **6**. Reaction of the compound of Formula **6**

with an eqimolar equivalent, based upon thiol, of a suitable nitrosylating agent, such as, thionyl chloride nitrite, thionyl dinitrite, a lower alkyl nitrite, such as, tert-butyl nitrite, or nitrosonium tetrafluoroborate in a suitable anhydrous solvent, such as, methylene chloride, THF, DMF, or acetonitrile, with or without an amine base, such as, pyridine or triethylamine affords the compound of Formula **7**. Alternatively, treatment of compound **6** with a stoichiometric quantity of sodium nitrite in an acidic aqueous or alcoholic solution affords the compound of Formula **7**. The compound of Formula **7** is then converted to the compound of Formula **IB** by deprotecting the amine (strong acid, such as, HCl in dioxane or trifluoroacetic acid is used to remove a t-butyl carbamate).

Nitro compounds of Formula (**I**), wherein R_e, R_f, k, and p are as defined herein, D^1 is hydrogen and a O-nitrosated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 3**. The compound of Formula **2**, wherein P^3 is as defined herein, with the preferred protecting group for the amine being a carbamate, such as, a t-butyl carbamate, is converted to the ester of Formula **8**, wherein p, R_e and R_f are as defined herein, by reaction with an appropriate nitrate containing activated acylating agent. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the nitrate containing acid or condensing the alcohol and nitrate containing acid in the presence of a dehydrating agent, such as, DCC or EDAC·HCl, with or without a catalyst, such as, DMAP or HOBt. Deprotection of the amine (strong acid, such as, HCl in dioxane or trifluoroacetic acid is used to remove a t-butyl carbamate) affords a compound of Formula **IC**.

2-Hydroxy-2-nitrosohydrazine compounds of Formula (**I**), wherein R_e, R_f, R_i, and p are as defined herein, D^1 is hydrogen and hydrogen and a 2-hydroxy-2-nitrosohydrazine ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 4**. The compound of Formula **2**, wherein P^3 is as defined herein, with the preferred protecting group for the amine being an amide, such as, a trifluoroacetamide, is converted to the ester of Formula **9**, wherein p, R_e, R_f and R_i are as defined herein, by reaction with an appropriate protected amine containing activated acylating agent wherein $P^{3'}$ is an amine protecting group. Preferred $P^{3'}$ protecting groups for the amine are as a carbamate, such as, a benzyl or tert-butyl carbamate. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected amine containing acid or condensing the alcohol and protected amine containing acid in the presence of a dehydrating agent, such as, DCC or EDAC·HCl, with or without a catalyst,

such as, DMAP or HOBt. Removal of the $P^{3'}$ amine protecting group (hydrogen in the presence of a transition metal catalyst, such as, palladium or platinum, is a preferred method for removing benzyl carbamate protecting groups, strong anhydrous acids, such as, trifluoroacetic acid or hydrochloric acid in methanol, dioxane or ethyl acetate are preferred for removing the t-butyl carbamate protecting group) followed by treatment of the amine with nitric oxide (1-5 atmospheres) in a dry inert solvent, such as, ether or tetrahydrofuran, affords the compound of Formula **10** wherein M^+, is as defined herein. The compound of Formula **10** is then converted to the compound of Formula **ID** by removing the remaining amine protecting group (mild base, such as, aqueous sodium or potassium carbonate or ammonia in methanol are the preferred methods for removing trifluoroacetamide protecting groups).

Nitroso compounds of Formula (II) wherein R_e, R_f, and p are as defined herein, $P^{1'}$ is an acetyl or trifluoroacetyl ester or a benzyl ether, and hydrogen and an O-nitrosylated ester are representative of the D groups as defined above may be prepared as outlined in **Figure 5**. An alcohol group of Formula **11** is converted to the ester of Formula **12** wherein p, R_e and R_f are as defined herein by reaction with an appropriate protected alcohol containing activated acylating agent, wherein P^1 is as defined herein. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected alcohol containing acid or condensing the alcohol and protected alcohol containing acid in the presence of a dehydrating agent, such as, DCC or EDAC·HCl, with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the alcohol moiety are silyl ethers, such as, a trimethylsilyl or tert-butyldimethylsilyl ether. Protection of the remaining secondary alcohol as an ester, such as, an acetyl or trifluoroacetyl ester, followed by deprotection of the silylated hydroxyl moiety (fluoride ion is the preferred method for removing silyl ether protecting groups) and then reaction a suitable nitrosylating agent, such as, thionyl chloride nitrite, thionyl dinitrite, or nitrosonium tetrafluoroborate, in a suitable anhydrous solvent, such as, dichloromethane, THF, DMF, or acetonitrile, with or without an amine base, such as, pyridine or triethylamine affords the compound of Formula **13**. The compound of Formula **13** is then converted to the compound of Formula **IIA** by deprotecting the remaining hydroxyl group. Hydrogen in the presence of a transition metal catalyst, such as, palladium or platinum, is a preferred method for removing the benzyl ether protecting group, and mild base, such as, aqueous sodium or potassium carbonate or ammonia in methanol, are the preferred methods for removing, trifluoroacetyl ester or

acetyl ester protecting groups.

Nitroso compounds of Formula (**II**) wherein R_e, R_f, and p are as defined herein, and hydrogen and a S-nitrosylated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 6**. The compound of Formula **11** is converted to the ester of Formula **14,** wherein p, R_e and R_f , are as defined herein, by reaction with an appropriate protected thiol containing activated acylating agent ,wherein P^2 is as defined herein. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected thiol containing acid or condensing the alcohol and protected thiol containing acid in the presence of a dehydrating agent, such as, DCC or EDAC · HCl, with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the thiol moiety are as a thioester, such as, a thioacetate or thiobenzoate, as a disulfide, as a thiocarbamate, such as, N-methoxymethyl thiocarbamate, or as a thioether, such as, a paramethoxybenzyl thioether, a tetrahydropyranyl thioether or a 2,4,6-trimethoxybenzyl thioether. Deprotection of the thiol moiety (zinc in dilute aqueous acid, triphenylphosphine in water and sodium borohydride are preferred methods for reducing disulfide groups. while aqueous base is typically utilized to hydrolyze thioesters and N-methoxymethyl thiocarbamates and mercuric trifluoroacetate, silver nitrate, or strong acids, such as, trifluoroacetic or hydrochloric acid and heat are used to remove a paramethoxybenzyl thioether, a tetrahydropyranyl thioether or a 2,4,6-trimethoxybenzyl thioether group) to afford a compound of Formula **15**. Reaction of the compound of Formula **15** with an equimolar equivalent (based upon thiol) of a suitable nitrosylating agent, such as, thionyl chloride nitrite, thionyl dinitrite, or a lower alkyl nitrite, such as, tert-butyl nitrite, or nitrosonium tetrafluoroborate in a suitable anhydrous solvent, such as, methylene chloride, THF, DMF, or acetonitrile, with or without an amine base, such as, pyridine or triethylamine, affords the compound of Formula **IIB**. Alternatively, treatment of compound **15** with a stoichiometric quantity of sodium nitrite in an acidic aqueous or alcoholic solution affords the compound of Formula **IIB**.

Nitro compounds of Formula (**II**), wherein R_e, R_f, k, and p are as defined here, and hydrogen and an O-nitrosated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 7**. The compound of Formula **11** is converted to the ester of Formula **IIC,** wherein p, k, R_e and R_f ,are as defined herein, by reaction with an appropriate nitrate containing activated acylating agent. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical

anhydride of the nitrate containing acid or condensing the alcohol and nitrate containing acid in the presence of a dehydrating agent, such as, DCC or EDAC·HCl, with or without a catalyst, such as, DMAP or HOBt.

2-Hydroxy-2-nitrosohydrazine compounds of Formula (**II**), wherein R_e, R_f, R_i, and p, are as defined herein, and hydrogen and a 2-hydroxy-2-nitrosohydrazine ester are representative of the D groups, as defined herein, may be prepared as outlined in **Figure 8**. The compound of Formula **11** is converted to the ester of Formula **16**, wherein p, R_i, R_e and R_f are as defined herein, by reaction with an appropriate protected amine containing activated acylating agent, wherein P^3 is an amine protecting group. Preferred protecting groups for the amine are as a carbamate, such as, a benzyl or tert-butyl carbamate. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected amine containing acid or condensing the alcohol and protected amine containing acid in the presence of a dehydrating agent, such as, DCC or EDAC·HCl, with or without a catalyst, such as, DMAP or HOBt. Removal of the P^3 amine protecting group (hydrogen in the presence of a transition metal catalyst, such as, palladium or platinum, is a preferred method for removing benzyl carbamate protecting groups, while strong anhydrous acids, such as, trifluoroacetic acid or hydrochloric acid in methanol, dioxane or ethyl acetate, are preferred for removing the t-butyl carbamate protecting group) followed by treatment of the amine with nitric oxide (1-5 atmospheres) in a dry inert solvent, such as, ether or tetrahydrofuran, affords the compound of Formula **IID**, wherein M^+ is as defined herein.

Nitroso compounds of Formula (**III**) wherein R_e, R_f, and p are as defined herein, $P^{1'}$ is an acetyl ester or a benzyl carbonate, and hydrogen and an O-nitrosylated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 9**. The alcohol and acid groups of Formula **17** are protected to afford the compound of Formula **18**. Preferred protecting groups for the alcohol are as a carbamate, such as, a benzyl carbonate or an ester, such as, a acetyl ester, while preferred protecting groups for the acids are as an ester, such as, t-butyl ester. Deprotection of the hydroxyl moiety (catalytic hydrogenation is the preferred method for cleaving benzyl carbonates while mild aqueous base removes the acetyl ester group) followed by reaction of the alcohol group with an appropriate protected alcohol containing activated acylating agent, wherein R_e, R_f, and p and P^1, are as defined herein, affords a compound of Formula **19**. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical

anhydride of the protected alcohol containing acid or condensing the alcohol and protected alcohol containing acid in the presence of a dehydrating agent, such as, DCC or EDAC . HCl, with or without, a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the alcohol moiety are silyl ethers, such as, a tert-butyldimethylsilyl ether. Deprotection of the acid and hydroxyl moieties (strong acid, such as, HCl in dioxane or trifluoroacetic acid cleaves t-butyl esters while fluoride ion is the preferred method for removing silyl ether protecting groups) followed by reaction a suitable nitrosylating agent, such as, thionyl chloride nitrite, thionyl dinitrite, or nitrosonium tetrafluoroborate in a suitable anhydrous solvent, such as, dichloromethane, THF, DMF, or acetonitrile with or without an amine base, such as, pyridine or triethylamine affords the compound of Formula **IIIA**.

Nitroso compounds of Formula (**III**) wherein R_e, R_f, and p are as defined herein, $P^{1'}$ is an acetyl ester or a benzyl carbonate, and hydrogen and an O-nitrosylated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 10**. The compound of Formula **18**, wherein the preferred protecting groups for the alcohol are as a carbonate, such as, a benzyl carbonate or an ester, such as, a acetyl ester, while a preferred protecting group for the acid is as an ester, such as, t-butyl ester, is converted to the compound of Formula **20** by removal of the t-butyl ester moiety (strong acid, such as, HCl in dioxane or trifluoroacetic acid cleaves t-butyl esters). The compound of Formula 20 is converted to the ester of Formula **21** by reaction of the acid group with an appropriate protected alcohol containing alcohol, wherein R_e, R_f, p and P^1 are as defined herein. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected alcohol containing acid or condensing the alcohol and protected alcohol containing acid in the presence of a dehydrating agent, such as, DCC or EDAC . HCl with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the alcohol moiety on the protected alcohol containing alcohol are silyl ethers, such as, tert-butyldimethylsilyl ether. Deprotection of the silyl hydroxyl moiety (fluoride ion is the preferred method for removing silyl ether protecting groups) followed by reaction a suitable nitrosylating agent, such as, thionyl chloride nitrite, thionyl dinitrite, or nitrosonium tetrafluoroborate in a suitable anhydrous solvent, such as, dichloromethane, THF, DMF, or acetonitrile, with or without an amine base, such as, pyridine or triethylamine, affords the compound of Formula **22**. Removal of the remaining hydroxyl protecting group (catalytic hydrogenation is the preferred method for cleaving benzyl carbonates while mild aqueous base removes the acetyl ester group)

affords the compound of Formula **IIIB**.

Nitroso compounds of Formula (**III**), wherein R_e, R_f, and p are as defined herein, and hydrogen and an S-nitrosylated ester are representative of the D group as defined herein, may be prepared as outlined in **Figure 11**. The compound of Formula **18**, wherein the preferred protecting groups for the alcohol are as a carbonate, such as, a benzyl carbonate or an ester, such as, a acetyl ester while preferred protecting groups for the acid is as an ester, such as, a t-butyl ester, is converted to the compound of Formula **23** by deprotection of the hydroxyl moiety (catalytic hydrogenation is the preferred method for cleaving benzyl carbonates while mild aqueous base removes the acetyl ester group). Reaction of the alcohol group with an appropriate protected thiol containing activated acylating agent, wherein R_e, R_f, and p and P^2, are as defined herein, afford the compound of Formula **24**. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected thiol containing acid or condensing the alcohol and protected thiol containing acid in the presence of a dehydrating agent, such as, DCC or EDAC ' HCl, with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the thiol moiety are as a thioester, such as, a thioacetate or thiobenzoate, as a disulfide, as a thiocarbamate, such as, N- methoxymethyl thiocarbamate, or as a thioether, such as, a paramethoxybenzyl thioether, a tetrahydropyranyl thioether or a 2,4,6-trimethoxybenzyl thioether. Deprotection of the thiol and acid moieties (zinc in dilute aqueous acid, triphenylphosphine in water and sodium borohydride are preferred methods for reducing disulfide groups, while aqueous base is typically utilized to hydrolyze thioesters and N-methoxymethyl thiocarbamates and mercuric trifluoroacetate, silver nitrate, or strong acids, such as, trifluoroacetic or hydrochloric acid and heat, are used to remove a paramethoxybenzyl thioether, a tetrahydropyranyl thioether, or a 2,4,6-trimethoxybenzyl thioether group as well as t-butyl esters) followed by reaction a suitable nitrosylating agent, such a,s thionyl chloride nitrite, thionyl dinitrite, a lower alkyl nitrite, such as, tert-butyl nitrite, or nitrosonium tetrafluoroborate in a suitable anhydrous solvent, such as, methylene chloride, THF, DMF, or acetonitrile, with or without an amine base, such as, pyridine or triethylamine, affords the compound of Formula **IIIC**. Alternatively, treatment of the deprotected compound with a stoichiometric quantity of sodium nitrite in an acidic aqueous or alcoholic solution affords the compound of Formula **IIIC**.

Nitroso compounds of Formula (**III**) wherein R_e, R_f, and p are as defined herein, $P^{1'}$ is an acetyl ester or a silyl ether, such as, trimethylsilyl ether or t-butyldimethylsilyl ether, and

hydrogen and an S-nitrosylated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 12**. The compound of Formula **20** is converted to the ester of Formula **25** by reaction of the acid group with an appropriate protected thiol containing alcohol wherein R_e, R_f, and p and P^2, are as defined herein. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected alcohol containing acid or condensing the alcohol and protected alcohol containing acid in the presence of a dehydrating agent, such as, DCC or EDAC.HCl, with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the thiol moiety are as a thioester, such as, a thioacetate or thiobenzoate, as a disulfide, as a thiocarbamate such as, N- methoxymethyl thiocarbamate, or as a thioether, such as, a paramethoxybenzyl thioether, a tetrahydropyranyl thioether or a 2,4,6-trimethoxybenzyl thioether. Deprotection of the thiol and alcohol moieties (zinc in dilute aqueous acid, triphenylphosphine in water and sodium borohydride are preferred methods for reducing disulfide groups while aqueous base is typically utilized to hydrolyze thioesters, esters and N-methoxymethyl thiocarbamates and mercuric trifluoroacetate, silver nitrate, or strong acids, such as, trifluoroacetic or hydrochloric acid and heat, are used to remove a paramethoxybenzyl thioether, a tetrahydropyranyl thioether, or a 2,4,6-trimethoxybenzyl thioether group, while fluoride is the preferred method for removing silyl ether protecting groups) followed by reaction a suitable nitrosylating agent, such as, thionyl chloride nitrite, thionyl dinitrite, a lower alkyl nitrite, such as, tert-butyl nitrite, or nitrosonium tetrafluoroborate, in a suitable anhydrous solvent, such as, methylene chloride, THF, DMF, or acetonitrile, with or without an amine base, such as, pyridine or triethylamine, affords the compound of Formula **IIID**. Alternatively, treatment of the deprotected compound with a stoichiometric quantity of sodium nitrite in an acidic aqueous or alcoholic solution affords the compound of Formula **IIID**.

Nitro compounds of Formula (**III**), wherein R_e, R_f, k, and p are as defined herein, and hydrogen and an O-nitrosated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 13**. The compound of Formula **23** is converted to the ester of Formula **26** wherein p, k, R_e and R_f are as defined herein, by reaction with an appropriate nitrate containing activated acylating agent. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the nitrate containing acid or condensing the alcohol and nitrate containing acid in the presence of a dehydrating agent, such as, DCC or EDAC·HCl, with or without a

catalyst, such as, DMAP or HOBt. Deprotection of the acid (strong acid, such as, HCl in dioxane or trifluoroacetic acid cleaves t-butyl esters) affords the compound of Formula **IIIE**.

Nitro compounds of Formula (**III**) wherein R_e, R_f, and p are as defined herein, and hydrogen and an O-nitrosated ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 14**. The compound of Formula **20**, wherein the preferred alcohol protecting group is an ester, such as, an acetyl ester or a silyl ether, such as, a trimethylsilyl of tert-butyldimethyl silyl ether is converted to the ester of Formula **27** wherein p, R_e and R_f are defined as herein, by reaction with an appropriate nitrate containing alcohol. Preferred methods for the formation of esters are reacting the nitrate containing alcohol with the preformed acid chloride or symmetrical anhydride or condensing the nitrate containing alcohol and acid in the presence of a dehydrating agent, such as, DCC or EDAC.HCl, with or without a catalyst, such as, DMAP or HOBt. Removal of the remaining hydroxyl protecting group (mild aqueous base removes the acetyl ester group while fluoride ion is the preferred method for removing silyl ether protecting groups) affords the compound of Formula **IIIF**.

2-Hydroxy-2-nitrosohydrazine compounds of Formula (**III**) wherein R_e, R_f, R_i, and p are as defined herein, and hydrogen and a 2-hydroxy-2-nitrosohydrazine ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 15.** The alcohol group of Formula **25** is converted to the ester of Formula **28** wherein p, R_e, R_f, R_i and P^3 are as defined herein, by reaction with an appropriate protected amine containing activated acylating agent. Preferred methods for the formation of esters are reacting the alcohol with the preformed acid chloride or symmetrical anhydride of the protected amino containing acid or condensing the alcohol and protected amine containing acid in the presence of a dehydrating agent, such as, DCC or EDAC.HCl, with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the amine are as a carbamate, such as, a t-butyl carbamate or a 9- fluorenylmethyl carbamate or an amide, such as, a trifluoroacetamide. Deprotection of the amino and t-butyl ester moieties (strong acid, such as, HCl in dioxane or trifluoroacetic acid, is used to remove a t-butyl carbamate as well as the t-butyl ester groups, while piperidine is used to remove 9-fluorenylmethyl carbamate, and mild aqueous or alcoholic base may be used to cleave a trifluoroacetamide group) followed by treatment of the amine with nitric oxide (1-5 atmospheres) in a dry inert solvent, such as, ether or tetrahydrofuran, affords the compound of Formula **IIIG** wherein

M^+ is as defined herein.

2-Hydroxy-2-nitrosohydrazine compounds of Formula **(III)** wherein R_e, R_f, R_i, and p are as defined herein. P^1 is preferably an acetyl ester or silyl protecting group, such as, trimethylsilyl ether or t-butyldimethylsilyl ether, and hydrogen and a 2-hydroxy-2-nitrosohydrazine ester are representative of the D groups as defined herein, may be prepared as outlined in **Figure 16**. The acid group of Formula **20** is converted to the ester of Formula **29** wherein p, R_e, R_f, R_i and P^3 are as defined herein, by reaction with an appropriate protected amine containing alcohol. Preferred methods for the formation of esters are reacting the protected amine containing alcohol with the preformed acid chloride or symmetrical anhydride of the acid or condensing the protected amine containing alcohol and acid in the presence of a dehydrating agent, such as, DCC or EDAC.HCl, with or without a catalyst, such as, DMAP or HOBt. Preferred protecting groups for the amine are as a carbamate, such as, a t-butyl carbamate or a 9- fluorenylmethyl carbamate or an amide, such as, a trifluoroacetamide. Deprotection of the amino and alcohol moieties (strong acid, such as, HCl in dioxane or trifluoroacetic acid, is used to remove a t-butyl carbamat, while piperidine is used to remove 9-fluorenylmethyl carbamate, while mild aqueous or alcoholic base may be used to cleave a acetyl ester group, and fluoride is used for removing silyl ethers) followed by treatment of the amine with nitric oxide (1-5 atmospheres) in a dry inert solvent, such as, ether or tetrahydrofuran affords the compound of Formula **IIIH**, wherein M^+ is as defined herein.

The nitrosated and/or nitrosylated nebivolol and the nitrosated and/or nitrosylated metabolites of nebivolol of the invention donate, transfer or release a biologically active form of nitrogen monoxide (nitric oxide). Nitrogen monoxide can exist in three forms: NO- (nitroxyl), NO• (nitric oxide) and NO^+ (nitrosonium). NO• is a highly reactive short-lived species that is potentially toxic to cells. This is critical because the pharmacological efficacy of NO depends upon the form in which it is delivered. In contrast to the nitric oxide radical (NO•), nitrosonium (NO^+) does not react with O_2 or O_2- species, and functionalities capable of transferring and/or releasing NO^+ and NO- are also resistant to decomposition in the presence of many redox metals. Consequently, administration of charged NO equivalents (positive and/or negative) does not result in the generation of toxic by-products or the elimination of the active NO moiety.

Compounds contemplated for use in the invention (e.g., nebivolol and/or nitrosated and/or nitrosylated nebivolol and/or metabolites of nebivolol and/or metabolites

of nitrosated and/or nitrosylated nebivolol) are, optionally, used in combination with nitric oxide and compounds that release nitric oxide or otherwise directly or indirectly deliver or transfer nitric oxide to a site of its activity, such as on a cell membrane *in vivo*.

The term "nitric oxide" encompasses uncharged nitric oxide (NO•) and charged nitrogen monoxide species, preferably charged nitrogen monoxide species, such as nitrosonium ion (NO^+) and nitroxyl ion (NO-). The reactive form of nitric oxide can be provided by gaseous nitric oxide. The nitrogen monoxide releasing, delivering or transferring compounds have the structure F-NO, wherein F is a nitrogen monoxide releasing, delivering or transferring moiety, and include any and all such compounds which provide nitrogen monoxide to its intended site of action in a form active for its intended purpose. The term "NO adducts" encompasses any nitrogen monoxide releasing, delivering or transferring compounds, including, for example, S-nitrosothiols, nitrites, nitrates, S-nitrothiols, sydnonimines, 2-hydroxy-2-nitrosohydrazines, (NONOates), (E)-alkyl-2-((E)- hydroxyimino)-5-nitro-3-hexeneamide (FK-409), (E)-alkyl-2-((E)-hydroxyimino)-5-nitro- 3-hexeneamines, N-((2Z,3E)-4-ethyl-2-(hydroxyimino)-6-methyl-5-nitro-3-heptenyl)- 3-pyridinecarboxamide (FR 146801), nitrosoamines, furoxans as well as substrates for the endogenous enzymes which synthesize nitric oxide. NONOates include, but are not limited to, (Z)-1-(N-methyl-N-(6-(N-methyl-ammoniohexyl)amino))diazen-1-ium-1,2-diolate ("MAHMA/NO"), (Z)-1-(N-(3-ammoniopropyl)-N-(n-propyl)amino)diazen-1-ium-1,2-diolate ("PAPA/NO"), (Z)-1-(N-(3-aminopropyl)-N-(4-(3-aminopropylammonio) butyl)-amino)diazen- 1-ium-1,2-diolate (spermine NONOate or "SPER/NO") and sodium (Z)-1-(N,N-diethylamino) diazenium-1,2-diolate (diethylamine NONOate or "DEA/NO") and derivatives thereof. NONOates are also described in U.S. Patent Nos. 6,232,336, 5,910,316 and 5,650,447, the disclosures of which are incorporated herein by reference in their entirety. The "NO adducts" can be mono-nitrosylated, poly-nitrosylated, mono-nitrosated and/or poly-nitrosated at a variety of naturally susceptible or artificially provided binding sites for biologically active forms of nitrogen monoxide.

One group of NO adducts is the S-nitrosothiols, which are compounds that include at least one -S-NO group. These compounds include S-nitroso-polypeptides (the term "polypeptide" includes proteins and polyamino acids that do not possess an ascertained biological function, and derivatives thereof); S-nitrosylated amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures and derivatives

thereof); S-nitrosylated sugars; S-nitrosylated, modified and unmodified, oligonucleotides (preferably of at least 5, and more preferably 5-200 nucleotides); straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted S-nitrosylated hydrocarbons; and S-nitroso heterocyclic compounds. S-nitrosothiols and methods for preparing them are described in U.S. Patent Nos. 5,380,758 and 5,703,073; WO 97/27749; WO 98/19672; and Oae et al, *Org. Prep. Proc. Int.*, *15*(3):165-198 (1983), the disclosures of each of which are incorporated by reference herein in their entirety.

Another embodiment of the invention is S-nitroso amino acids where the nitroso group is linked to a sulfur group of a sulfur-containing amino acid or derivative thereof. Such compounds include, for example, S-nitroso-N-acetylcysteine, S-nitroso-captopril, S-nitroso-N- acetylpenicillamine, S-nitroso-homocysteine, S-nitroso-cysteine, S-nitroso-glutathione, S-nitroso- cysteinyl-glycine, and the like.

Suitable S-nitrosylated proteins include thiol-containing proteins (where the NO group is attached to one or more sulfur groups on an amino acid or amino acid derivative thereof) from various functional classes including enzymes, such as tissue-type plasminogen activator (TPA) and cathepsin B; transport proteins, such as lipoproteins; heme proteins, such as hemoglobin and serum albumin; and biologically protective proteins, such as immunoglobulins, antibodies and cytokines. Such nitrosylated proteins are described in WO 93/09806, the disclosure of which is incorporated by reference herein in its entirety. Examples include polynitrosylated albumin where one or more thiol or other nucleophilic centers in the protein are modified.

Other examples of suitable S-nitrosothiols include:

(i) $HS(C(R_e)(R_f))_mSNO$;

(ii) $ONS(C(R_e)(R_f))_mR_e$; and

(iii) $H_2N\text{-}CH(CO_2H)\text{-}(CH_2)_m\text{-}C(O)NH\text{-}CH(CH_2SNO)\text{-}C(O)NH\text{-}CH_2\text{-}CO_2H$;

wherein m is an integer from 2 to 20; R_e and R_f are each independently a hydrogen, an alkyl, a cycloalkoxy, a halogen, a hydroxy, an hydroxyalkyl, an alkoxyalkyl, an arylheterocyclic ring, an alkylaryl, an alkylcycloalkyl, an alkylheterocyclic ring, a cycloalkylalkyl, a cycloalkylthio, a cycloalkenyl, an heterocyclicalkyl, an alkoxy, a haloalkoxy, an amino, an alkylamino, a dialkylamino, an arylamino, a diarylamino, an alkylarylamino, an alkoxyhaloalkyl, a haloalkoxy, a sulfonic acid, a sulfonic ester, an alkylsulfonic acid, an arylsulfonic acid, an arylalkoxy, an alkylthio, an arylthio, a cyano an aminoalkyl, an aminoaryl, an aryl, an arylalkyl, an alkylaryl, a carboxamido, a

alkylcarboxamido, an arylcarboxamido, an amidyl, a carboxyl, a carbamoyl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarbonyl, an arylcarbonyl, an ester, a carboxylic ester, an alkylcarboxylic ester, an arylcarboxylic ester, a haloalkoxy, a sulfonamido, an alkylsulfonamido, an arylsulfonamido, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfonyl, arylsulphonyloxy, a sulfonic ester, a urea, a phosphoryl, a nitro, W_h, -T-Q , or $-(C(R_e)(R_f))_k$-T-Q, or R_e and R_f taken together with the carbons to which they are attached form a carbonyl, a methanthial, a heterocyclic ring, a cycloalkyl group, an aryl group, an oxime or a bridged cycloalkyl group; Q is -NO or $-NO_2$; and T is independently a covalent bond, a carbonyl, an oxygen, $-S(O)_o$- or $-N(R_a)R_i$-, wherein o is an integer from 0 to 2, R_a is a lone pair of electrons, a hydrogen or an alkyl group; R_i is a hydrogen, an alkyl, an aryl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarboxylic ester, an arylcarboxylic ester, an alkylcarboxamido, an arylcarboxamido, an alkylaryl, an alkylsulfinyl, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfinyl, an arylsulfonyl, arylsulphonyloxy, a sulfonamido, a carboxamido, a carboxylic ester, an aminoalkyl, an aminoaryl, $-CH_2-C(T-Q)(R_e)(R_f)$, a bond to an adjacent atom creating a double bond to that atom, $-(N_2O_2-)^-\bullet M^+$, wherein M^+ is an organic or inorganic cation; with the proviso that when R_i is $-CH_2-C(T-Q)(R_e)(R_f)$ or $-(N_2O_2-)\bullet M^+$; then "-T-Q" can be a hydrogen, an alkyl group, an alkoxyalkyl group, an aminoalkyl group, a hydroxy group or an aryl group.

In cases where R_e and R_f are a heterocyclic ring or R_e and R_f taken together with the hetero atom to which they are attached are a heterocyclic ring, then R_i can be a substituent on any disubstituted nitrogen contained within the radical wherein R_i is as defined herein.

Nitrosothiols can be prepared by various methods of synthesis. In general, the thiol precursor is prepared first, then converted to the S-nitrosothiol derivative by nitrosation of the thiol group with $NaNO_2$ under acidic conditions (pH is about 2.5) which yields the S-nitroso derivative. Acids which can be used for this purpose include aqueous sulfuric, acetic and hydrochloric acids. The thiol precursor can also be nitrosylated by reaction with an organic nitrite such as tert-butyl nitrite, or a nitrosonium salt such as nitrosonium tetrafluoroborate in an inert solvent.

Another group of NO adducts for use in the invention, where the NO adduct is a compound that donates, transfers or releases nitric oxide, include compounds comprising at least one ON-O-, ON-N- or ON-C- group. The compounds that include at least one ON-O-, ON-N- or ON-C- group are preferably ON-O-, ON-N- or ON-C-polypeptides (the term

"polypeptide" includes proteins and polyamino acids that do not possess an ascertained biological function, and derivatives thereof); ON-O-, ON-N- or ON-C-amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures); ON-O-, ON-N- or ON-C-sugars; ON-O-, ON-N- or ON-C- modified or unmodified oligonucleotides (comprising at least 5 nucleotides, preferably 5-200 nucleotides); ON-O-, ON-N- or ON-C- straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted hydrocarbons; and ON-O-, ON-N- or ON-C-heterocyclic compounds.

Another group of NO adducts for use in the invention include nitrates that donate, transfer or release nitric oxide, such as compounds comprising at least one O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- group. Preferred among these compounds are O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures); O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C-sugars; O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- modified and unmodified oligonucleotides (comprising at least 5 nucleotides, preferably 5-200 nucleotides); O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted hydrocarbons; and O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- heterocyclic compounds. Preferred examples of compounds comprising at least one O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- group include isosorbide dinitrate, isosorbide mononitrate, clonitrate, erythrityl tetranitrate, mannitol hexanitrate, nitroglycerin, pentaerythritoltetranitrate, pentrinitrol, propatylnitrate and organic nitrates with a sulfhydryl-containing amino acid such as, for example SPM 3672, SPM 5185, SPM 5186 and those disclosed in U. S. Patent Nos. 5,284,872, 5,428,061, 5,661,129, 5,807,847 and 5,883,122 and in U.S. Provisional Application No. 60/311,175 and in WO 97/46521 and WO 00/54756, the disclosures of each of which are incorporated by reference herein in their entirety.

Another group of NO adducts are N-oxo-N-nitrosoamines that donate, transfer or release nitric oxide and are represented by the Formula: R^1R^2N-N(O-M^+)-NO, where R^1 and R^2 are each independently a polypeptide, an amino acid, a sugar, a modified or unmodified oligonucleotide, a straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted hydrocarbon, or a heterocyclic group, and where M^+ is an

organic or inorganic cation, such as, for example, an alkyl substituted ammonium cation or a Group I metal cation.

Another group of NO adducts are thionitrates that donate, transfer or release nitric oxide and are represented by the formula: R^1-(S)-NO_2, where R^1 is a polypeptide, an amino acid, a sugar, a modified or unmodified oligonucleotide, a straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted hydrocarbon, or a heterocyclic group. Preferred are those compounds where R^1 is a polypeptide or hydrocarbon with a pair or pairs of thiols that are sufficiently structurally proximate, i.e., vicinal, that the pair of thiols will be reduced to a disulfide. Compounds which form disulfide species release nitroxyl ion (NO-) and uncharged nitric oxide (NO•).

The invention is also directed to compounds that stimulate endogenous NO or elevate levels of endogenous endothelium-derived relaxing factor (EDRF) *in vivo* or are substrates for the enzyme, nitric oxide synthase. Such compounds include, for example, L-arginine, L-homoarginine, and N-hydroxy-L-arginine, including their nitrosated and nitrosylated analogs (e.g., nitrosated L-arginine, nitrosylated L-arginine, nitrosated N-hydroxy-L-arginine, nitrosylated N-hydroxy-L-arginine, nitrosated L-homoarginine and nitrosylated L-homoarginine), precursors of L-arginine and/or physiologically acceptable salts thereof, including, for example, citrulline, ornithine, glutamine, lysine, polypeptides comprising at least one of these amino acids, inhibitors of the enzyme arginase (e.g., N-hydroxy-L-arginine and 2(S)-amino-6-boronohexanoic acid) and the substrates for nitric oxide synthase, cytokines, adenosin, bradykinin, calreticulin, bisacodyl, and phenolphthalein. EDRF is a vascular relaxing factor secreted by the endothelium, and has been identified as nitric oxide (NO) or a closely related derivative thereof (Palmer et al, *Nature*, 327:524-526 (1987); Ignarro et al, *Proc. Natl. Acad. Sci. USA*, 84:9265-9269 (1987)).

The invention is also based on the discovery that the administration of a therapeutically effective amount of the compounds and compositions described herein is effective for treating and/or preventing vascular diseases characterized by nitric oxide (NO) insufficiency. For example, the patient can be administered a therapeutically effective amount of at least one nitrosated and/or nitrosylated nebivolol of the invention. In another embodiment, the patient can be administered a therapeutically effective amount of at least one nitrosated and/or nitrosylated metabolite of nebivolol. In yet another embodiment, the patient can be administered a therapeutically effective amount of nebivolol, optionally

substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and at least one compound that donates, transfers or releases nitric oxide as a charged species, or elevates levels of endogenous EDRF or nitric oxide, or is a substrate for nitric oxide synthase. In another embodiment, the patient can be administered a therapeutically effective amount of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and, optionally, at least one compound that donates, transfers or releases nitric oxide as a charged species, or elevates levels of endogenous EDRF or nitric oxide, or is a substrate for nitric oxide synthase and/or at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one compound used to treat cardiovascular diseases, or a pharmaceutically acceptable salt thereof. The compound used to treat cardiovascular diseases can optionally be substituted with at least one NO_2 group (i.e. nitrosated). The compounds can be administered separately or as a composition.

In the invention the compound that donates, transfers or releases nitric oxide as a charged species, or elevates levels of endogenous EDRF or nitric oxide, or is a substrate for nitric oxide synthase may preferably be isosorbide dinitrate and/or isosorbide mononitrate, more preferably isosorbide dinitrate. Diluted isosorbide dinitrate(1,4,3,6-dianhydro-D-glucitol-2,5-dinitrate), USP, is a white to off-white powder that has a melting point of 70 °C and has an optical rotation of +135° (3 mg/mL, ethanol). It is freely soluble in organic solvents such as ethanol, ether and chloroform, but is sparingly soluble in water. Isosorbide dinitrate is commercially available, for example, under the trade names DILATRATE®-SR (Schwarz Pharma, Milwaukee, WI); ISORDIL® and ISORDILR TITRADOSE® (Wyeth Laboratories Inc., Philadelphia, PA); and SORBITRATE® (Zeneca Pharmaceuticals, Wilmington, DE). Isosorbide mononitrate is commercially available, for example, under the trade names IMDUR® (A. B. Astra, Sweden); MONOKET® (Schwarz Pharma, Milwaukee, WI); and ISMO® (Wyeth-Ayerst company, Philadelphia, PA).

In the invention, the antioxidants include small-molecule antioxidants and antioxidant enzymes. Antioxidant refers to and includes any compound that can react and quench a free radical. Suitable small-molecule antioxidants include, but are not limited to, hydralazine compounds, glutathione, vitamin C, vitamin E, cysteine, N-acetyl-cysteine, β-carotene, ubiquinone, ubiquinol-10, tocopherols, coenzyme Q, and the like. Suitable antioxidant enzymes include, but are not limited to, superoxide dismutase, catalase,

glutathione peroxidase, and the like. Suitable antioxidants are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file reg. The preferred antioxidant is a hydralazine compound that may preferably be administered as a pharmaceutically acceptable salt; more preferably as hydralazine hydrochloride. Hydralazine hydrochloride is commercially available from, for example, Lederle Standard Products (Pearl River, NY), and Par Pharmaceuticals Inc. (Spring Valley, NY).

The compound used to treat cardiovascular diseases, or a pharmaceutically acceptable salt, include, but are not limited to, angiotensin-converting enzyme (ACE) inhibitors, beta-adrenergic blockers, cholesterol reducers, calcium channel blockers, angiotensin II receptor antagonists, endothelin antagonists, renin inhibitors, and the like, and mixtures thereof.

Suitable angiotensin-converting enzyme inhibitors, include, but are not limited to, alacepril, benazepril, captopril, ceronapril, cilazapril, delapril, duinapril, enalapril, enalaprilat, fosinopril, imidapril, lisinopril, losartan, moveltipril, naphthopidil, pentopril, perindopril, quinapril, ramipril, rentipril, spirapril, temocapril, trandolapril, urapidil, zofenopril, and the like. Suitable angiotensin-converting enzyme inhibitors are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file registry.

Suitable beta-adrenergic blockers, include, but are not limited to, acebutolol, alprenolol, amosulalol, arotinolol, atenolol, betaxolol, bethanidine, bevantolol, bisoprolol, bopindolol, bucumolol, bufetolol, bufuralol, bunitrolol, bupranolol, butafilolol, carazolol, carteolol, carvedilol, celiprolol, cetamolol, dilevalol, epanolol, esmolol, indenolol, labetalol, mepindolol, metipranolol, metoprolol, moprolol, nadolol, nadoxolol, nifenalol, nipradilol, oxprenolol, penbutolol, pindolol, practolol, pronethalol, propranolol, sotalol, sulfinalol, talinolol, tertatolol, tilisolol, timolol, toliprolol, xibenolol, and the like. Suitable beta-adrenergic blockers are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file registry.

Suitable cholesterol reducers include but are not limited to HMG-CoA reductase

inhibitors, such as, for example, lovastatin (MEVACOR®), simvastatin (ZOCOR®), pravastatin (PRAVACHOL®), fluvastatin, cerivastatin (BAYCOL®), atorvastatin (LIPITOR®), and the like; sequestrants such as, for example, cholestyramine, colestipol, sialkylaminoalkyl derivatives of cross-linked dextran, and the like; inhibitors of cholesterol absorption, such as, for example, beta-sitosterol, acyl CoA-cholersterol acyltransferase inhibitors, melinamide, and the like. Suitable calcium channel blockers are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file registry.

Suitable calcium channel blockers, include, but are not limited to, amlodipine, aranidipine, barnidipine, benidipine, cilnidipine, clentiazem, diltiazen, efonidipine, fantofarone, felodipine, isradipine, lacidipine, lercanidipine, manidipine, mibefradil, nicardipine, nifedipine, nilvadipine, nisoldipine, nitrendipine, semotiadil, veraparmil, and the like. Suitable calcium channel blockers are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file registry.

Suitable endothelin antagonists, include, but are not limited to, bosentan, sulfonamide endothelin antagonists, BQ-123, SQ 28608, and the like. Suitable endothelin antagonists are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file registry.

Suitable angiotensin II receptor antagonists, include, but are not limited to, ciclosidomine, eprosartan, furosemide, irbesartan, losartan, saralasin, valsartan, and the like. Suitable angiotensin II receptor antagonists are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file registry.

Suitable renin inhibitors, include, but are not limited to, enalkrein, RO 42-5892, A 65317, CP 80794, ES 1005, ES 8891, SQ 34017, and the like). Suitable renin inhibitors are described more fully in the literature, such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995; and the Merck

Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; and on STN Express, file phar and file registry.

The compound used to treat cardiovascular diseases, or a pharmaceutically acceptable salt, can be nitrosated through one or more sites such as oxygen (hydroxyl condensation), sulfur (sulfhydryl condensation), and/or nitrogen. The nitrosated angiotensin-converting enzyme inhibitors, nitrosated beta-adrenergic blockers, nitrosated cholesterol reducer, nitrosated calcium channel blockers, nitrosated endothelin antagonists, nitrosated angiotensin II receptor antagonists and nitrosated renin inhibitors of the invention include any known angiotensin-converting enzyme inhibitors, beta-adrenergic blockers, cholesterol reducer, calcium channel blockers, endothelin antagonists, angiotensin II receptor antagonists and renin inhibitors that have been nitrosated through one or more sites such as oxygen (hydroxyl condensation), sulfur (sulfhydryl condensation), and/or nitrogen. The nitrosated compounds of the invention can be prepared using conventional methods known to one skilled in the art. For example, known methods for nitrosating compounds are described in U.S. Patent Nos. 5,380,758 and 5,703,073; WO 97/27749; WO 98/19672; and Oae et al, *Org. Prep. Proc. Int.*, *15*(3):165-198 (1983), the disclosures of each of which are incorporated by reference herein in their entirety. WO 98/21193 discloses nitrosated ACE inhibitors and nitrosated beta-adrenergic blockers, the disclosure of which is incorporated by reference herein in its entirety. WO 99/00361 discloses nitrate salts of ACE inhibitors, the disclosure of which is incorporated by reference herein in its entirety.

In addition to the administration of the combination of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and at least one compound that donates, transfers or releases nitric oxide as a charged species, or elevates levels of endogenous EDRF or nitric oxide, or is a substrate for nitric oxide synthase and the antioxidant and/or the compound used to treat cardiovascular diseases, for the treatment of vascular diseases characterized by nitric oxide insufficiency, the patients can receive digitalis such as digoxin and/or diuretics.

The digoxin may preferably be administered orally to achieve a steady state blood serum concentration of at least about 0.7 nanograms per ml to about 2.0 nanograms per ml. The diuretic is administered, preferably orally, to manage edema. Suitable diuretics include, but are not limited to, thiazides (such as, for example, chlorothiazide, hydrochlorothiazide); ethacrynic acid, furosemide, spironalactone, triamterene or mixtures

thereof. Depending on the diuretic used, potassium may also be administered to the patient in order to optimize the fluid balance while avoiding hypokalemic alkalosis. The administration of potassium can be as potassium chloride or by the daily ingestion of foods with high potassium content such as, for example, bananas, orange juice, and the like. The method of administration of these compounds is described in further detail in U.S. Patent No. 4,868,179, the disclosure of which is incorporated by reference herein in its entirety.

The invention also provides methods of preventing and treating Raynaud's syndrome by administering a therapeutically effective amount of at least one nebivolol, optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and, optionally, at least one compound that donates, transfers or releases nitric oxide as a charged species, or elevates levels of endogenous EDRF or nitric oxide, or is a substrate for nitric oxide synthase and/or at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one nitrosated compound used to treat cardiovascular diseases such as, for example, nitrosated angiotensin-converting enzyme inhibitor, nitrosated beta-adrenergic blocker, nitrosated cholesterol reducer, nitrosated calcium channel blocker, nitrosated endothelin antagonist, nitrosated angiotensin II receptor antagonist and/or nitrosated renin inhibitor. For example, the patient can be administered a therapeutically effective amount of at least one nitrosated and/or nitrosylated nebivolol of the invention. In another embodiment, the patient can be administered a therapeutically effective amount of at least one nitrosated and/or nitrosylated metabolite of nebivolol. In yet another embodiment, the patient can be administered a therapeutically effective amount of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and at least one compound that donates, transfers or releases nitric oxide as a charged species, or elevates levels of endogenous EDRF or nitric oxide, or is a substrate for nitric oxide synthase. In another embodiment, the patient can be administered a therapeutically effective amount of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and, optionally, at least one compound that donates, transfers or releases nitric oxide as a charged species, or elevates levels of endogenous EDRF or nitric oxide, or is a substrate for nitric oxide synthase and/or at least one antioxidant or a pharmaceutically acceptable salt thereof, and/or at least one nitrosated compound used to treat cardiovascular diseases. For example, the

patient can be administered a nitrosated and/or nitrosylated nebivolol, a nitric oxide donor and an antioxidant, or the patient can be administered a nitrosated and/or nitrosylated metabolite of nebivolol, a nitric oxide donor and an antioxidant, or the patient can be administered nebivolol, a nitric oxide donor and an antioxidant. The nebivolol, nitric oxide donor, antioxidant and nitrosated compound used to treat cardiovascular diseases can be administered separately or as components of the same composition. Raynaud's syndrome is a condition that causes a loss of blood flow to the fingers, toes, nose and/or ears. The affected area turns white from the lack of circulation, then blue and cold, and finally numb. The affected area may also turn red, and may throb, tingle or swell.

In the methods of the invention, nebivolol, optionally substituted with at least one NO and/or NO_2 group, the metabolites of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and, optionally, nitric oxide donor, antioxidant and/or compound used to treat cardiovascular diseases, optionally substituted with at least one NO_2 group, can be administered as separate components or as components of the same composition. When the nebivolol, optionally substituted with at least one NO and/or NO_2 group, metabolite of nebivolol, optionally substituted with at least one NO and/or NO_2 group, and, optionally, nitric oxide donor, antioxidant, and/or compound used to treat cardiovascular diseases, optionally substituted with at least one NO_2 group, are administered as separate components for the treatment of vascular diseases characterized by nitric oxide insufficiency or Raynaud's syndrome, they are preferably administered to the patient at about the same time. "About the same time" includes after administering one compound (e.g., nebivolol or metabolite of nebivolol or nitric oxide donor or antioxidant or compound used to treat cardiovascular diseases) to the patient, the other compound (e.g., nitric oxide donor or antioxidant or compound used to treat cardiovascular diseases or nebivolol or metabolite of nebivolol) is administered to the patient. "About the same time" also includes simultaneous administration of the compounds or administering the compounds at the same time, at different times on the same day, or on different days, as long as they are administered as part of an overall treatment regimen.

Another embodiment of the invention provides compositions comprising nebivolol, optionally substituted with at least one NO and/or NO_2 group, and/or at least one metabolite of nebivolol, that are optionally nitrosated and/or nitrosylated, and, optionally, at least one compound that donates, transfers or releases nitric oxide and/or stimulates the endogenous production of NO or EDRF *in vivo* and/or is a substrate for nitric oxide synthase and/or at

least one therapeutic agent and/or at least one nitrosated and/or nitrosylated therapeutic agent, bound to a matrix.

The nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and, optionally, NO donors and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, can be incorporated into a natural or synthetic matrix which can then be applied with specificity to a biological site of interest. Accordingly the optionally substituted nebivolol and/or metabolite of nebivolol, and, optionally, NO donor is "bound to the matrix" which means that the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, NO donors and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, are physically and/or chemically associated with part of, incorporated with, attached to, or contained within the natural or synthetic matrix. In one embodiment, physical association or bonding can be achieved, for example, by coprecipitation of the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, NO donor and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, with the matrix. In another embodiment, chemical association or bonding can be achieved by, for example, covalent bonding of a nucleophilic moiety of the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, NO donor and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, to the matrix, such that nebivolol and/or metabolite of nebivolol is part of the matrix itself. In yet another embodiment, the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, NO donor and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, can be incorporated into a porous layer of the matrix or into pores included in the natural or synthetic matrix. The manner in which the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, NO donor and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, is associated, part of, attached to, incorporated with or contained within (i.e. "bound to") the matrix is inconsequential to the invention and all means of association, incorporation, attachment, and bonding are contemplated herein. Incorporation of the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, NO donors and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, into the matrix results in site-specific application, thereby

enhancing selectivity of action for the released nitric oxide and nebivolol and/or metabolite of nebivolol. Additionally, incorporation of the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol into the matrix reduces the rate of release of the nitric oxide and nebivolol and/or metabolite of nebivolol. This prolongs the release of the nitric oxide and nebivolol and/or metabolite of nebivolol thereby allowing for efficient dosing to achieve a desired biological effect so that the frequency of dosing can be reduced.

Any of a wide variety of natural or synthetic polymers can be used as the matrix in the context of the invention. It is only necessary for the matrix to be biologically acceptable. Exemplary matrixes suitable for use in the invention are polymers including, for example, polyolefins (such as polystyrene, polypropylene, polyethylene, high density polyethylene, polytetrafluorethylene, polyvinylidene diflouride and polyvinylchloride), polyethylenimine or derivatives thereof, polyethers (such as polyethylene glycol), polyesters (such as poly-L-lactic acid, poly-D, L-lactic, poly-D-lactic, polyglycolic, poly-(lactide/glycolide)), polyanhydrides, polyhydroxybutyrates, polyamides (such as nylon), polyurethanes, polyurethane copolymers (such as pellethane polymers), polyacrylates (such as polymethacrylate, poly (2-(methacryloyloxyethyl)-2'-(trimethylammonium)ethyl phosphate inner salt-co-n-dodecyl methacrylate), mixtures of polymers (such as polylactic acid/polylysine copolymers, polyurethane/polyester copolymers, polyurethane/polyether copolymers, nylon/polyether copolymers, such as vestamid), biopolymers (such as peptides, proteins, oligonucleotides, antibodies, peptide hormones, glycoproteins, glycogen and nucleic acids), starburst dendrimers, natural fibrous matrix (such as filter paper), synthetic fibrous matrix materials (such as three-dimensional lattice of synthetic polymers and copolymers) and the like. Exemplary polymers are described in U. S. Patent Nos. 5,705,583, 5,770,645 and 5,994,444 and Application Serial No. 08/460,465, the disclosures of which are incorporated by reference herein in their entirety.

The physical and structural characteristics of the matrixes suitable for use in the invention are not critical, but depend on the application. It will be appreciated by one skilled in the art that where the matrix-nebivolol and/or matrix-metabolite of nebivolol composition of the invention is intended for local, relatively short term administration or similar administration they need not be biodegradable. For some uses, such as postangioplasty, coronary bypass surgery or intimal hyperplasia associated with vascular graft implants or the like, it may be desirable for the matrix to slowly dissolve in a physiological environment

or to be biodegradable or bioresorbable.

The nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol and, optionally, the compound that donates, transfers or releases nitric oxide and/or stimulates the endogenous production of NO or EDRF *in vivo* and/or is a substrate for nitric oxide synthase and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, bound to the matrix may be administered in a wide variety of forms or delivery means. Any delivery means should adequately protect the integrity of the nitric oxide prior to its release and should control the release of the nitric oxide at such a rate, in such an amount, and in such a location as to serve as an effective means for prevention and/or treatment of cardiovascular diseases or disorders, including restenosis. Delivery means for local administration include, for example, sutures, vascular implants, stents, heart valves, drug pumps, drug delivery catheters and the like. Delivery means for systemic administration include, for example, solutions, suspensions, emulsions, capsules, powders, sachets, tablets, effervescent tablets, topical patches, lozenges, aerosols, liposomes, microparticles, microspheres, beads and the like. The matrix itself may be structurally sufficient to serve as a delivery means.

The nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol and, optionally, the compound that donates, transfers or releases nitric oxide and/or stimulates the endogenous production of NO or EDRF *in vivo* and/or is a substrate for nitric oxide synthase, and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, bound to the matrix can also be used to coat the surface of a medical device or instrument that comes into contact with blood (including blood components and blood products) or vascular tissue thereby rendering the surface passive. Alternatively the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol and the compound that donates, transfers or releases nitric oxide and/or stimulates the endogenous production of NO or EDRF *in vivo* and/or is a substrate for nitric oxide synthase, and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, bound to the matrix can also be used to coat the surface of a medical device or instrument that comes into contact with blood (including blood components and blood products) or vascular tissue thereby rendering the surface passive. U.S. Patent Nos. 5,837,008, 5,665,077, 5,797,887 and 5,824,049, the disclosures of each of which are incorporated by reference herein in their entirety, describe methods for coating a surface of a medical device or instrument. Thus, for example, (i) all or a portion of

the medical device may be coated with the nitrosated and/or nitrosylated nebivolol, and, optionally, NO donors and/or therapeutic agents and/or nitrosated and/or nitrosylated therapeutic agents, either as the coating *per se* or bound to a matrix, as described herein; or (ii) all or a portion of the medical device may be produced from a material which includes the nitrosated and/or nitrosylated nebivolol, and, optionally, NO donor, therapeutic agent and nitrosated and/or nitrosylated therapeutic agent, *per se* or bound to a matrix, as described herein.

It is also contemplated that artificial surfaces will vary depending on the nature of the surface, and such characteristics including contour, crystallinity, hydrophobicity, hydrophilicity, capacity for hydrogen bonding, and flexibility of the molecular backbone and polymers. Therefore, using routine methods, one of ordinary skill will be able to customize the coating technique by adjusting such parameters as the amount of adduct, length of treatment, temperature, diluents, and storage conditions, in order to provide optimal coating of each particular type of surface.

After the device or artificial material has been coated with the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol, and, optionally, NO donor, and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, or with nebivolol and/or metabolite of nebivolol and NO donor, and, optionally, therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, it will be suitable for its intended use, including, for example, implantation as a heart valve, insertion as a catheter, insertion as a stent, or for cardiopulmonary oxygenation or hemodialysis.

Therapeutic agents useful in the invention include, but is not limited to, agents which biologically stent a vessel and/or reduce or inhibit vascular remodeling and/or inhibit or reduce vascular smooth muscle proliferation following a procedural vascular trauma. The "therapeutic agents" of the invention include agents that inhibit the cellular activity of a vascular smooth muscle cell, for example, proliferation, migration, increase in cell volume, increase in extracellular matrix synthesis (e.g., collagens, proteoglycans, and the like), or secretion of extracellular matrix materials by the cell. Suitable "therapeutic agents" include, but are not limited to, antithrombogenic agents (such as, for example, heparin, covalent heparin, hirudin, hirulog, coumadin, protamine, argatroban, D-phenylalanyl-L-poly-L-arginyl chloromethyl ketone, and the like); thrombolytic agents (such as, for example, urokinase, streptokinase, tissueplasminogen activators, and the like); fibrinolytic agents;

vasospasm inhibitors; potassium channel activators (such as, for example, nicorandil, pinacidil, cromakalim, minoxidil, aprilkalim, loprazolam and the like); calcium channel blockers (such as, for example, nifedipine, veraparmil, diltiazem, gallopamil, niludipine, nimodipins, nicardipine, and the like); antihypertensive agents (such as, for example, HYTRIN®, and the like); antimicrobial agents or antibiotics (such as, for example, adriamycin, and the like); antiplatelet agents (such as, for example, aspirin, ticlopidine, a glycoprotein IIb/IIIa inhibitor, surface glycoprotein receptors and the like); antimitotic, antiproliferative agents or microtubule inhibitors (such as, for example, taxanes, colchicine, methotrexate, azathioprine, vincristine, vinblastine, cytochalasin, fluorouracil, adriamycin, mutamycin, tubercidin, epothilone A or B, discodermolide, and the like); antisecretory agents (such as, for example, retinoid, and the like); remodelling inhibitors; antisense nucleotides (such as, for example, deoxyribonucleic acid, and the like); anti-cancer agents (such as, for example, tamoxifen citrate, acivicin, bizelesin, daunorubicin, epirubicin, mitoxantrone, and the like); steroids (such as, for example, dexamethasone, dexamethasone sodium phosphate, dexamethasone acetate, and the like); non-steroidal antiinflammatory agents (NSAID); COX-2 inhibitors; immunosuppressive agents (such as, for example cyclosporin, rapamycin, everolimus, actinomycin D and the like); growth factor antagonists or antibodies (such as, for example, trapidal (a PDGF antagonist), angiopeptin (a growth hormone antagonist), angiogenin, and the like); dopamine agonists (such as, for example, apomorphine, bromocriptine, testosterone, cocaine, strychnine, and the like); radiotherapeutic agents (such as, for example, ^{60}Co (5.3 year half life), ^{192}Ir (73.8 days), ^{32}P (14.3 days), ^{111}In (68 hours), ^{90}Y (64 hours), ^{99m}Tc (6 hours), and the like); heavy metals functioning as radiopaque agents (such as, for example, iodine-containing compounds, barium-containing compounds, gold, tantalum, platinum, tungsten, and the like); biologic agents (such as, for example, peptides, proteins, enzymes, extracellular matrix components, cellular components, and the like); angiotensin converting enzyme (ACE) inhibitors; angiotensin II receptor antagonists; renin inhibitiors; free radical scavengers, iron chelators or antioxidants (such as, for example, ascorbic acid, alpha tocopherol, superoxide dismutase, deferoxamine, 21-aminosteroid, and the like); sex hormones (such as, for example, estrogen, and the like); antipolymerases (such as, for example, AZT, and the like); antiviral agents (such as, for example, acyclovir, famciclovir, rimantadine hydrochloride, ganciclovir sodium, Norvir®, Crixivan®, and the like); photodynamic therapy agents (such as, for example, 5-aminolevulinic acid, meta-tetrahydroxyphenylchlorin, hexadecafluoro

zinc phthalocyanine, tetramethyl hematoporphyrin, rhodamine 123, and the like); antibody targeted therapy agents (such as, for example, IgG2 Kappa antibodies against Pseudomonas aeruginosa exotoxin A and reactive with A431 epidermoid carcinoma cells, monoclonal antibody against the noradrenergic enzyme dopamine beta-hydroxylase conjugated to saporin, and the like); and gene therapy agent. Preferred therapeutic agents, include antiproliferative agents, such as, for example, taxanes; steroids such as, for example, dexamethasone, immunosuppressive agents, such as for example, rapamycin, everolimus, actinomycin D and the like. The therapeutic agent can optionally be substituted with at least one NO and/or NO_2 group (i.e., nitrosylated and/or nitrosated) through one or more sites such as oxygen (hydroxyl condensation), sulfur (sulfhydryl condensation), and/or nitrogen. The compounds and compositions of the invention can also be administered in combination with other medications used for the treatment of these diseases or disorders.

Suitable taxanes, include, but are not limited to, for example, paclitaxel and docetaxel, water soluble compositions of paclitaxel and docetaxel, pro-drugs of paclitaxel and docetaxel, as well as functional analogs, equivalents or derivatives of taxanes, and the like. For example, derivatives and analogs of taxanes include, but are not limited to, baccatin III, 10-deacetyltaxol, 7-xylosyl-10-deacetyltaxol, cephalomannine, 10-deacetyl-7-epitaxol, 7-epitaxol, 10-deacetylbaccatin III, 10-deacetylcephaolmannine and analogs or derivatives, and the like. Taxanes are disclosed in, for example, U. S. Patent Nos. 4,960,790, 5,157,049, 5,284,864, 5,399,726, 5,550,261, 5,616,608, 5,629,433, 5,646,176, 5,688,977, 5,703,117, 5,760,072, 5,808,113, 5,912,263, 5,919,815, 5,965,739, 5,977,163, 5,981,564, 5,998,656, 6,017,935, 6,017,948, 6,028,205 and in WO 93/17121, WO 94/15599, WO 95/20582, WO 96/00724, WO 96/40091, WO 97/10234, WO 97/19938, WO 97/32578, WO 97/33552, WO 98/00419, WO 98/28288, WO 98/37765, WO 98/38862, WO 99/14209, WO 99/49901, WO 99/57105, WO 00/10988 and in EP 0 558 959 B1, EP 0 624 377 A2, EP 0 639 577 A1, the disclosures of each of which are incorporated by reference herein in their entirety. Taxanes and their nitrosating and/or nitrosylated derivatives are also disclosed in U. S. Application No. 09/886,494, assigned to NitroMed Inc.; and in WO 00/61537, WO 00/61541 and WO 01/12584; the disclosure of each of which are incorporated by reference herein in its entirety.

Suitable anticoagulants include, but are not limited to, heparin, coumarin, aspirin, protamine, warfarin, dicumarol, phenprocoumon, indan-1,3-dione, acenocoumarol, ansindione, and the like. Suitable anticoagulants are described more fully in the literature,

such as in Goodman and Gilman, The Pharmacological Basis of Therapeutics (9th Edition), McGraw-Hill, 1995, Pgs. 1341-1359; the Merck Index on CD-ROM, Twelfth Edition, Version 12:1, 1996; STN express file reg and file phar.

Another embodiment of the invention provides methods for the prevention of platelet aggregation and platelet adhesion caused by the exposure of blood (including blood components or blood products) to a medical device or instrument by incorporating at least one nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol, and, optionally, at least one compound that donates, transfers or releases nitric oxide and/or stimulates the endogenous production of NO or EDRF *in vivo* and/or is a substrate for nitric oxide synthase, and/or therapeutic agent and/or nitrosated and/or nitrosylated therapeutic agent, capable of releasing a therapeutically effective amount of nitric oxide, into and/or on the portion(s) of the medical device that come into contact with blood (including blood components or blood products) or vascular tissue. The nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol, and, optionally, NO donors, therapeutic agents and/or nitrosated and/or nitrosylated therapeutic agents, may be directly or indirectly linked to the natural or synthetic polymeric material from which all or a portion of the device is made, as disclosed in U. S. Patent Nos. 6,087,479 and 6,174,539, assigned to NitroMed, the disclosure of each of which are incorporated by reference herein in its entirety. Alternatively, the nitrosated and/or nitrosylated nebivolol, and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol, and, optionally, NO donors, therapeutic agents and/or nitrosated and/or nitrosylated therapeutic agents, may be incorporated into the body of the device that is formed of a biodegradable or bioresorbable material, including the matrix described herein. Thus the nitric oxide is released over a sustained period of the resorption or degradation of the body of the device.

Another embodiment of the invention relates to local administration of the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol, and, optionally, at least one compound that donates, transfers or releases nitric oxide and/or stimulates the endogenous production of NO or EDRF *in vivo* and/or is a substrate for nitric oxide synthase, and/or at least one therapeutic agent and/or at least one nitrosated and/or nitrosylated therapeutic agent, to the site of injured or damaged tissue (e.g., damaged blood vessels) for the treatment of the injured or damaged tissue. Such damage may result from the use of a medical device in an invasive procedure. Thus, for

example, in treating blocked vasculature by, for example, angioplasty, damage can result to the blood vessel. Such damage may be treated by use of the compounds and compositions described herein. In addition to repair of the damaged tissue, such treatment can also be used to prevent and/or alleviate and/or delay re-occlusions, for example, restenosis. The compounds and compositions can be locally delivered using any of the methods known to one skilled in the art, including but not limited to, a drug delivery catheter, an infusion catheter, a drug delivery guidewire, an implantable medical device, and the like. In one embodiment, all or most of the damaged area is coated with the nitrosated and/or nitrosylated nebivolol described herein *per se* or in a pharmaceutically acceptable carrier or excipient which serves as a coating matrix, including the matrix described herein. This coating matrix can be of a liquid, gel or semisolid consistency. The carrier or matrix can be made of or include agents which provide for metered or sustained release of the therapeutic agents.

In preventing and/or treating cardiovascular diseases or disorders, the nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol, and, optionally, at least one compound that donates, transfers or releases nitric oxide and/or stimulates the endogenous production of NO or EDRF *in vivo* and/or is a substrate for nitric oxide synthase and/or at least one therapeutic agent and/or at least one nitrosated and/or nitrosylated therapeutic agent, can be administered directly to the damaged vascular surface intravenously by using an intraarterial or intravenous catheter, suitable for delivery of the compounds to the desired location. The location of damaged arterial surfaces is determined by conventional diagnostic methods, such as X-ray angiography, performed using routine and well-known methods available to one skilled in the art. In addition, administration of the nitrosated and/or nitrosylated nebivolol, and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol, and, optionally, NO donors, therapeutic agents and/or nitrosated and/or nitrosylated therapeutic agents, using an intraarterial or intravenous catheter is performed using routine methods well known to one skilled in the art. Typically, the compound or composition is delivered to the site of angioplasty through the same catheter used for the primary procedure, usually introduced to the carotid or coronary artery at the time of angioplasty balloon inflation. The nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolite of nebivolol and/or nebivolol, and, optionally, NO donors, therapeutic agents and nitrosated and/or nitrosylated therapeutic agents, slowly decompose at body temperature over a prolonged

period of time releasing nitric oxide at a rate effective to prevent and/or treat cardiovascular diseases or disorders including, for example, restenosis.

When administered *in vivo*, the compounds and compositions of the invention can be administered in combination with pharmaceutically acceptable carriers and in dosages described herein. When the compounds and compositions of the invention are administered as a mixture of at least one nitrosated and/or nitrosylated nebivolol or at least one nitrosated and/or nitrosylated metabolite of nebivolol or nebivolol or at least one metabolite of nebivolol and at least one nitric oxide donor, or at least one therapeutic agent or at least one nitrosated and/or nitrosylated therapeutic agent, they can also be used in combination with one or more additional therapeutic agents which are known to be effective against the specific disease state targeted for treatment. The nitric oxide donors and/or therapeutic agents can be administered simultaneously with, subsequently to, or prior to administration of nebivolol, including those that are substituted with one or more NO and/or NO_2 groups, and/or other additional compounds.

The compounds and compositions of the invention can be administered by any available and effective delivery system including, but not limited to, orally, bucally, parenterally, by inhalation spray, by topical application, by injection, transdermally, or rectally (e.g., by the use of suppositories) in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles, as desired. Parenteral includes subcutaneous injections, intravenous, intramuscular, intrasternal injection, or infusion techniques.

Topical compound administration, which is known to one skilled in the art, involves the delivery of pharmaceutical compounds via percutaneous passage of the compound into the systemic circulation of the patient. Topical administration can also involve the use of transdermal administration such as transdermal patches or iontophoresis devices. Other components can be incorporated into the transdermal patches as well. For example, compositions and/or transdermal patches can be formulated with one or more preservatives or bacteriostatic agents including, but not limited to, methyl hydroxybenzoate, propyl hydroxybenzoate, chlorocresol, benzalkonium chloride, and the like. Dosage forms for topical administration of the compounds and compositions can include creams, pastes, sprays, lotions, gels, ointments, eye drops, nose drops, ear drops, and the like. In such dosage forms, the compositions of the invention can be mixed to form white, smooth, homogeneous, opaque cream or lotion with, for example, benzyl alcohol 1% or 2% (wt/wt)

as a preservative, emulsifying wax, glycerin, isopropyl palmitate, lactic acid, purified water and sorbitol solution. In addition, the compositions can contain polyethylene glycol 400. They can be mixed to form ointments with, for example, benzyl alcohol 2% (wt/wt) as preservative, white petrolatum, emulsifying wax, and tenox II (butylated hydroxyanisole, propyl gallate, citric acid, propylene glycol). Woven pads or rolls of bandaging material, e.g., gauze, can be impregnated with the compositions in solution, lotion, cream, ointment or other such form can also be used for topical application. The compositions can also be applied topically using a transdermal system, such as one of an acrylic-based polymer adhesive with a resinous crosslinking agent impregnated with the composition and laminated to an impermeable backing.

Solid dosage forms for oral administration can include capsules, tablets, effervescent tablets, sustain release tablets, sustain release capsules, chewable tablets, pills, powders, sachets, granules and gels. In such solid dosage forms, the active compounds can be admixed with at least one inert diluent such as sucrose, lactose or starch. Such dosage forms can also comprise, as in normal practice, additional substances other than inert diluents, e.g., lubricating agents such as magnesium stearate. In the case of capsules, tablets, effervescent tablets, and pills, the dosage forms can also comprise buffering agents. Soft gelatin capsules can be prepared to contain a mixture of the active compounds or compositions of the invention and vegetable oil. Hard gelatin capsules can contain granules of the active compound in combination with a solid, pulverulent carrier such as lactose, saccharose, sorbitol, mannitol, potato starch, corn starch, amylopectin, cellulose derivatives of gelatin. Tablets and pills can be prepared with enteric coatings.

Liquid dosage forms for oral administration can include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions can also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

Suppositories for vaginal or rectal administration of the compounds and compositions of the invention can be prepared by mixing the compounds or compositions with a suitable nonirritating excipient such as cocoa butter and polyethylene glycols which are solid at room temperature but liquid at body temperature, such that they will melt and release the drug.

Injectable preparations, for example, sterile injectable aqueous or oleaginous

suspensions can be formulated according to the known art using suitable dispersing agents, wetting agents and/or suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be used are water, Ringer's solution, and isotonic sodium chloride solution. Sterile fixed oils are also conventionally used as a solvent or suspending medium.

The compositions of this invention can further include conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral application which do not deleteriously react with the active compounds. Suitable pharmaceutically acceptable carriers include, for example, water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, surfactants, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, and the like. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds. For parenteral application, particularly suitable vehicles consist of solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants. Aqueous suspensions may contain substances that increase the viscosity of the suspension and include, for example, sodium carboxymethyl cellulose, sorbitol and/or dextran. Optionally, the suspension may also contain stabilizers.

Solvents useful in the practice of this invention include pharmaceutically acceptable, water-miscible, non-aqueous solvents. In the context of this invention, these solvents should be taken to include solvents that are generally acceptable for pharmaceutical use, substantially water-miscible, and substantially non-aqueous. Preferably, these solvents are also non-phthalate plasticizer leaching solvents, so that, when used in medical equipment, they substantially do not leach phthalate plasticizers that may be present in the medical equipment. More preferably, the pharmaceutically-acceptable, water-miscible, non-aqueous solvents usable in the practice of this invention include, but are not limited to, N-methyl pyrrolidone (NMP); propylene glycol; ethyl acetate; dimethyl sulfoxide; dimethyl acetamide; benzyl alcohol; 2-pyrrolidone; benzyl benzoate; C_{2-6} alkanols; 2-ethoxyethanol; alkyl esters such as 2-ethoxyethyl acetate, methyl acetate, ethyl acetate, ethylene glycol

diethyl ether, or ethylene glycol dimethyl ether; (S)-(-)-ethyl lactate; acetone; glycerol; alkyl ketones such as methylethyl ketone or dimethyl sulfone; tetrahydrofuran; cyclic alkyl amides such as caprolactam; decylmethylsulfoxide; oleic acid; aromatic amines such as N,N-diethyl-m-toluamide; or 1-dodecylazacycloheptan-2-one.

The most preferred pharmaceutically-acceptable, water-miscible, non-aqueous solvents are N-methyl pyrrolidone (NMP), propylene glycol, ethyl acetate, dimethyl sulfoxide, dimethyl acetamide, benzyl alcohol, 2-pyrrolidone, or benzyl benzoate. Ethanol may also be used as a pharmaceutically-acceptable, water-miscible, non-aqueous solvent according to the invention, despite its negative impact on stability. Additionally, triacetin may also be used as a pharmaceutically-acceptable, water-miscible, non-aqueous solvent, as well as functioning as a solubilizer in certain circumstances. NMP may be available as PHARMASOLVE® from International Specialty Products (Wayne, N.J.). Benzyl alcohol may be available from J. T. Baker, Inc. Ethanol may be available from Spectrum, Inc. Triacetin may be available from Mallinkrodt, Inc.

The compositions of this invention can further include solubilizers. Solubilization is a phenomenon that enables the formation of a solution. It is related to the presence of amphiphiles, that is, those molecules that have the dual properties of being both polar and non-polar in the solution that have the ability to increase the solubility of materials that are normally insoluble or only slightly soluble, in the dispersion medium. Solubilizers often have surfactant properties. Their function may be to enhance the solubility of a solute in a solution, rather than acting as a solvent, although in exceptional circumstances, a single compound may have both solubilizing and solvent characteristics. Solubilizers useful in the practice of this invention include, but are not limited to, triacetin, polyethylene glycols (such as, for example, PEG 300, PEG 400, or their blend with 3350, and the like), polysorbates (such as, for example, Polysorbate 20, Polysorbate 40, Polysorbate 60, Polysorbate 65, Polysorbate 80, and the like), poloxamers (such as, for example, Poloxamer 124, Poloxamer 188, Poloxamer 237, Poloxamer 338, Poloxamer 407, and the like), polyoxyethylene ethers (such as, for example, Polyoxyl 2 cetyl ether, Polyoxyl 10 cetyl ether, and Polyoxyl 20 cetyl ether, Polyoxyl 4 lauryl ether, Polyoxyl 23 lauryl ether, Polyoxyl 2 oleyl ether, Polyoxyl 10 oleyl ether, Polyoxyl 20 oleyl ether, Polyoxyl 2 stearyl ether, Polyoxyl 10 stearyl ether, Polyoxyl 20 stearyl ether, Polyoxyl 100 stearyl ether, and the like), polyoxylstearates (such as, for example, Polyoxyl 30 stearate, Polyoxyl 40 stearate, Polyoxyl 50 stearate, Polyoxyl 100 stearate, and the like), polyethoxylated stearates (such as, for example, polyethoxylated

12-hydroxy stearate, and the like), and Tributyrin.

Other materials that may be added to the compositions of the invention include cyclodextrins, and cyclodextrin analogs and derivatives, and other soluble excipients that could enhance the stability of the inventive composition, maintain the product in solution, or prevent side effects associated with the administration of the inventive composition. Cyclodextrins may be available as ENCAPSIN® from Janssen Pharmaceuticals.

The composition, if desired, can also contain minor amounts of wetting agents, emulsifying agents and/or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like.

Various delivery systems are known and can be used to administer the compounds or compositions of the invention, including, for example, encapsulation in liposomes, microbubbles, emulsions, microparticles, microcapsules, nanoparticles, and the like. The required dosage can be administered as a single unit or in a sustained release form.

The bioavailabilty of the compositions can be enhanced by micronization of the formulations using conventional techniques such as grinding, milling, spray drying and the like in the presence of suitable excipients or agents such as phospholipids or surfactants.

Sustained release dosage forms of the invention may comprise microparticles and/or nanoparticles having a therapeutic agent dispersed therein or may comprise the therapeutic agent in pure, preferably crystalline, solid form. For sustained release administration, microparticle dosage forms comprising pure, preferably crystalline, therapeutic agents are preferred. The therapeutic dosage forms of this aspect of the invention may be of any configuration suitable for sustained release. Preferred sustained release therapeutic dosage forms exhibit one or more of the following characteristics: microparticles (e.g., from about 0.5 micrometers to about 100 micrometers in diameter, preferably about 0.5 to about 2 micrometers; or from about 0.01 micrometers to about 200 micrometers in diameter, preferably from about 0.5 to about 50 micrometers, and more preferably from about 2 to about 15 micrometers) or nanoparticles (e.g., from about 1.0 nanometer to about 1000 nanometers in diameter, preferably about 50 to about 250 nanometers ; or from about 0.01 nanometer to about 1000 nanometers in diameter, preferably from about 50 to about 200

nanometers), free flowing powder structure; biodegradable structure designed to biodegrade over a period of time between from about 0.5 to about 180 days, preferably from about 1 to 3 to about 150 days, more preferably from about 3 to about 180 days, and most preferably from about 10 to about 21 days; or non-biodegradable structure to allow the therapeutic agent diffusion to occur over a time period of between from about 0.5 to about 180 days, more preferably from about 30 to about 120 days; or from about 3 to about 180 days, more preferably from about 10 to about 21 days; biocompatible with target tissue and the local physiological environment into which the dosage form to be administered, including yielding biocompatible biodegradation products; facilitate a stable and reproducible dispersion of therapeutic agent therein, preferably to form a therapeutic agent-polymer matrix, with active therapeutic agent release occurring by one or both of the following routes: (1) diffusion of the therapeutic agent through the dosage form (when the therapeutic agent is soluble in the shaped polymer or polymer mixture defining the dimensions of the dosage form); or (2) release of the therapeutic agent as the dosage form biodegrades; and/or for targeted dosage forms, capability to have, preferably, from about 1 to about 10,000 binding protein/peptide to dosage form bonds and more preferably, a maximum of about 1 binding peptide to dosage form bond per 150 square angstroms of particle surface area. The total number of binding protein/peptide to dosage form bonds depends upon the particle size used. The binding proteins or peptides are capable of coupling to the particles of the therapeutic dosage form through covalent ligand sandwich or non-covalent modalities as set forth herein.

Nanoparticle sustained release therapeutic dosage forms are preferably biodegradable and, optionally, bind to the vascular smooth muscle cells and enter those cells, primarily by endocytosis. The biodegradation of the nanoparticles occurs over time (e.g., 30 to 120 days; or 10 to 21 days) in prelysosomic vesicles and lysosomes. Preferred larger microparticle therapeutic dosage forms of the invention release the therapeutic agents for subsequent target cell uptake with only a few of the smaller microparticles entering the cell by phagocytosis. A practitioner in the art will appreciate that the precise mechanism by which a target cell assimilates and metabolizes a dosage form of the invention depends on the morphology, physiology and metabolic processes of those cells. The size of the particle sustained release therapeutic dosage forms is also important with respect to the mode of cellular assimilation. For example, the smaller nanoparticles can flow with the interstitial fluid between cells and penetrate the infused tissue. The larger microparticles tend to be

more easily trapped interstitially in the infused primary tissue, and thus are useful to deliver anti-proliferative therapeutic agents.

Preferred sustained release dosage forms of the invention comprise biodegradable microparticles or nanoparticles. More preferably, biodegradable microparticles or nanoparticles are formed of a polymer containing matrix that biodegrades by random, nonenzymatic, hydrolytic scissioning to release therapeutic agent, thereby forming pores within the particulate structure.

The compounds and compositions of the invention can be formulated as pharmaceutically acceptable salts. Pharmaceutically acceptable salts include, for example, alkali metal salts and addition salts of free acids or free bases. The nature of the salt is not critical, provided that it is pharmaceutically-acceptable. Suitable pharmaceutically-acceptable acid addition salts may be prepared from an inorganic acid or from an organic acid. Examples of such inorganic acids include, but are not limited to, hydrochloric, hydrobromic, hydroiodic, nitrous (nitrite salt), nitric (nitrate salt), carbonic, sulfuric, phosphoric acid, and the like. Appropriate organic acids include, but are not limited to, aliphatic, cycloaliphatic, aromatic, heterocyclic, carboxylic and sulfonic classes of organic acids, such as, for example, formic, acetic, propionic, succinic, glycolic, gluconic, lactic, malic, tartaric, citric, ascorbic, glucuronic, maleic, fumaric, pyruvic, aspartic, glutamic, benzoic, anthranilic, mesylic, salicylic, p-hydroxybenzoic, phenylacetic, mandelic, embonic (pamoic), methanesulfonic, ethanesulfonic, benzenesulfonic, pantothenic, toluenesulfonic, 2-hydroxyethanesuifonic, sulfanilic, stearic, algenic, β-hydroxybutyric, cyclohexylaminosulfonic, galactaric and galacturonic acid and the like. Suitable pharmaceutically-acceptable base addition salts include, but are not limited to, metallic salts made from aluminum, calcium, lithium, magnesium, potassium, sodium and zinc or organic salts made from primary, secondary and tertiary amines, cyclic amines, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, meglumine (N-methylglucamine) and procaine and the like. All of these salts may be prepared by conventional means from the corresponding compound by reacting, for example, the appropriate acid or base with the compound.

While individual needs may vary, determination of optimal ranges for effective amounts of the compounds and/or compositions is within the skill of the art. Generally, the dosage required to provide an effective amount of the compounds and compositions, which can be adjusted by one of ordinary skill in the art, will vary depending on the age, health,

physical condition, sex, diet, weight, extent of the dysfunction of the recipient, frequency of treatment and the nature and scope of the dysfunction or disease, medical condition of the patient, the route of administration, pharmacological considerations such as the activity, efficacy, pharmacokinetic and toxicology profiles of the particular compound used, whether a drug delivery system is used, and whether the compound is administered as part of a drug combination.

The usual doses of nebivolol (including nitrosated and/or nitrosylated nebivolol, nitrosated and/or nitrosylated metabolites of nebivolol, and metabolites of nebivolol) for the treating and/or preventing vascular diseases characterized by nitric oxide insufficiency; and for treating and/or preventing Raynaud's syndrome is approximately 0.1 mg to about 10 mg per day, preferably about 5 mg per day, administered as a single dose once a day; in multiple doses several times throughout the day; or in a sustained-release formulation or as a transdermal patch.

The doses of nitric oxide donors in the pharmaceutical composition will be dependent on the specific nitric oxide donor compound and the mode of administration. For example, when isosorbide dinitrate is the orally administered nitric oxide donor, it can be administered in an amount of about 5 milligrams per day to about 200 milligrams per day. In a more particular embodiment, the isosorbide dinitrate can be administered in an amount of about 20 milligrams per day to about 160 milligrams per day. In an even more particular embodiment, the isosorbide dinitrate can be administered in an amount of about 40 milligrams one to four times per day. When isosorbide mononitrate is the orally administered nitric oxide donor, it can be administered in an amount of about 5 milligrams per day to about 120 milligrams per day. In a more particular embodiment, the isosorbide mononitrate can be administered in an amount of about 15 milligrams per day to about 100 milligrams per day. In an even more particular embodiment, the isosorbide mononitrate can be administered in an amount of about 20 milligrams one to four times per day. The particular amounts of isosorbide dinitrate and/or isosorbide mononitrate can be administered as a single dose once a day; or in multiple doses several times throughout the day; or as a sustained-release oral formulation; or as a transdermal sustained release patch.

The dose of nitric oxide donor in the composition will be dependent on the specific nitric oxide donor compound and the mode of administration. For example, when L-arginine is the orally administered nitric oxide donor, it can be administered in an amount of about 3 grams to about 15 grams to provide a plasma level in the range of about 0.2 mM to about 30

mM.

The doses of the antioxidant in the pharmaceutical composition will be dependent on the specific antioxidant compound and the mode of administration. For example when hydralazine is the administered antioxidant, it can be administered in an amount of about 30 milligrams per day to about 400 milligrams per day. In a more particular embodiment, the hydralazine hydrochloride can be administered in an amount of about 50 milligrams per day to about 300 milligrams per day. In an even more particular embodiment, the hydralazine hydrochloride can be administered in an amount of about 75 milligrams once to four times per day. The particular amounts of hydralazine can be administered as a single dose once a day; or in multiple doses several times throughout the day; or as a sustained-release oral formulation; or as a transdermal sustained release patch.

The nitrosated and/or nitrosylated nebivolol and/or nitrosated and/or nitrosylated metabolites of nebivolol of the invention are used at dose ranges and over a course of dose regimen and are administered in the same or substantially equivalent vehicles/carrier by the same or substantially equivalent as their non-nitrosated/nitrosylated counterparts. The nitrosated and/or nitrosylated compounds of the invention can also be used in lower doses and in less extensive regimens of treatment. The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration, and is within the skill in the art.

The invention also provides pharmaceutical kits comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compounds and/or compositions of the invention, including, nebivolol, optionally substituted with at least one NO and/or NO_2 group, one or more metabolites of nebivolol, optionally substituted with one or more NO and/or NO_2 groups, and one or more of the NO donors, and one or more antioxidants described herein. Such kits can also include, for example, other compounds and/or compositions (e.g., diuretics, digoxin, compounds used to treat cardiovascular diseases, therapeutic agents, permeation enhancers, lubricants, and the like), a device(s) for administering the compounds and/or compositions, and written instructions in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflects approval by the agency of manufacture, use or sale for human administration.

The disclosure of each patent, patent application and publication cited or described

in the specification is hereby incorporated by reference herein in its entirety.

Although the invention has been set forth in detail, one skilled in the art will appreciate that numerous changes and modifications may be made without departing from the spirit and scope of the invention.

5

CLAIMS

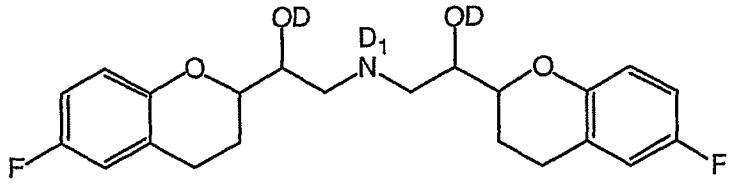
What is claimed is:

1. Nebivolol and/or a metabolite of nebivolol, or a stereoisomer thereof, having at least one NO group, at least one NO_2 group, or at least one NO and NO_2 group, or a pharmaceutically acceptable salt thereof.

2. Nebivolol and/or a metabolite of nebivolol, or a stereoisomer thereof, having at least one NO group or at least one NO and NO_2 group, or a pharmaceutically acceptable salt thereof.

3. A compound of Formula (I), Formula (II), Formula (III), Formula (IV) or Formula (V), an isomer thereof or a pharmaceutically acceptable salt thereof:

wherein the compound of Formula (I) is:

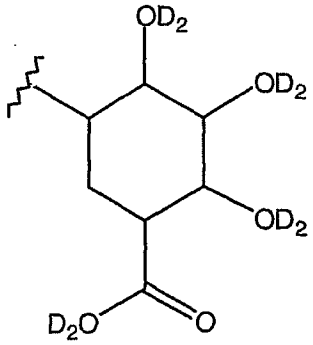


I

wherein:

D is hydrogen, Q, K or R_5;

R_5 is:



D_1 is hydrogen or R_5;

D_2 is hydrogen, Q or K;

Q is –NO or -NO_2;

K is $-W_a-E_b-(C(R_e)(R_f))_p-E_c-(C(R_e)(R_f))_x-W_d-(C(R_e)(R_f))_y-W_i-E_j-W_g-(C(R_e)(R_f))_z-$ T-Q;

a, b, c, d, g, i and j are each independently an integer from 0 to 3;

p, x, y and z are each independently an integer from 0 to 10;

W at each occurrence is independently -C(O)-, -C(S)-, -T-, $-(C(R_e)(R_f))_h-$, an alkyl group, an aryl group, a heterocyclic ring, an arylheterocyclic ring, or $-(CH_2CH_2O)_q-$;

E at each occurrence is independently -T-, an alkyl group, an aryl group, $-(C(R_e)(R_f))_h-$, a heterocyclic ring, an arylheterocyclic ring, or $-(CH_2CH_2O)_q-$;

h is an integer form 1 to 10;

q is an integer from 1 to 5;

R_e and R_f are each independently a hydrogen, an alkyl, a cycloalkoxy, a halogen, a hydroxy, an hydroxyalkyl, an alkoxyalkyl, an arylheterocyclic ring, an alkylaryl, an alkylcycloalkyl, an alkylheterocyclic ring, a cycloalkylalkyl, a cycloalkylthio, a cycloalkenyl, an heterocyclicalkyl, an alkoxy, a haloalkoxy, an amino, an alkylamino, a dialkylamino, an arylamino, a diarylamino, an alkylarylamino, an alkoxyhaloalkyl, a haloalkoxy, a sulfonic acid, a sulfonic ester, an alkylsulfonic acid, an arylsulfonic acid, an arylalkoxy, an alkylthio, an arylthio, a cyano an aminoalkyl, an aminoaryl, an aryl, an arylalkyl, an alkylaryl, a carboxamido, a alkylcarboxamido, an arylcarboxamido, an amidyl, a carboxyl, a carbamoyl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarbonyl, an arylcarbonyl, an ester, a carboxylic ester, an alkylcarboxylic ester, an arylcarboxylic ester, a haloalkoxy, a sulfonamido, an alkylsulfonamido, an arylsulfonamido, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfonyl, arylsulphonyloxy, a sulfonic ester, a urea, a phosphoryl, a nitro, W_h, -T-Q , or $-(C(R_e)(R_f))_k$-T-Q, or R_e and R_f taken together with the carbons to which they are attached form a carbonyl, a methanthial, a heterocyclic ring, a cycloalkyl group, an aryl group, an oxime or a bridged cycloalkyl group;

k is an integer from 1 to 3;

T at each occurrence is independently a covalent bond, a carbonyl, an oxygen, $-S(O)_o-$ or $-N(R_a)R_i-$;

o is an integer from 0 to 2;

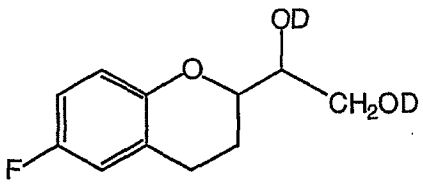
R_a is a lone pair of electrons, a hydrogen or an alkyl group;

R_i is a hydrogen, an alkyl, an aryl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarboxylic ester, an arylcarboxylic ester, an alkylcarboxamido, an arylcarboxamido, an alkylaryl, an alkylsulfinyl, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfinyl, an

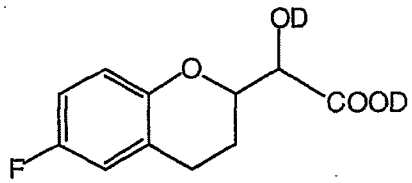
arylsulfonyl, arylsulphonyloxy, a sulfonamido, a carboxamido, a carboxylic ester, an aminoalkyl, an aminoaryl, $-CH_2-C(T-Q)(R_e)(R_f)$, a bond to an adjacent atom creating a double bond to that atom, $-(N_2O_2-)^-\bullet M^+$, wherein M^+ is an organic or inorganic cation;

with the proviso that the compound of Formula (I) must contain at least one nitrite, nitrate, thionitrite or thionitrate group;

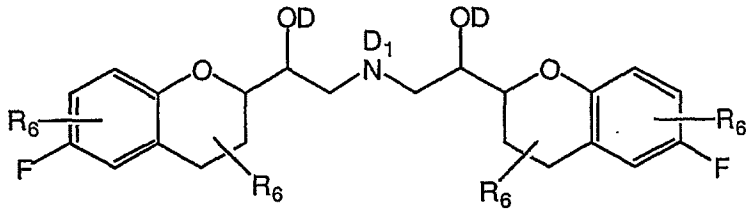
wherein the compounds of Formula (II), Formula (III), Formula (IV) and Formula (V) are:



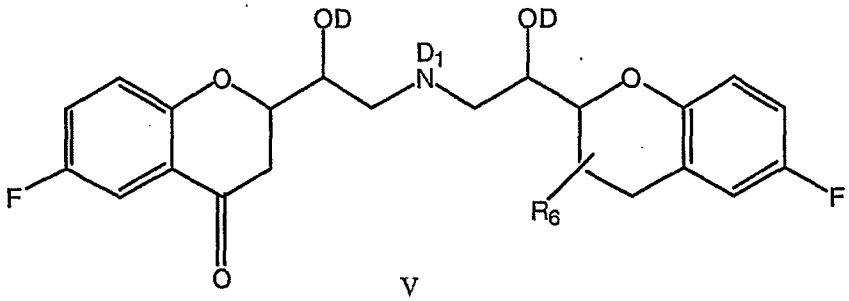
II



III



IV



V

wherein:

R_6 at each occurrence is independently a hydrogen, a hydroxy or -OD;

D and D_1 are as defined herein; and

with the proviso that the compounds of Formula (II), Formula (III), Formula (IV) and Formula (V), must contain at least one nitrite, nitrate, thionitrite or thionitrate group.

4. The compound of claim 3, wherein the compound of Formula (I) is a nitrosated nebivolol, a nitrosylated nebivolol, or a nitrosated and nitrosylated nebivolol, wherein the compounds of Formula (II), Formula (III), Formula (IV) and Formula (V) are a nitrosated metabolite of nebivolol, a nitrosylated metabolite of nebivolol, or a nitrosated and nitrosylated metabolite of nebivolol.

5. A composition comprising the compound of claim 3 and a pharmaceutically acceptable carrier.

6. A method of treating and/or preventing a vascular disease characterized by nitric oxide insufficiency in a patient in need thereof comprising administering a therapeutically effective amount of the composition of claim 5.

7. The method of claim 6, wherein the vascular disease characterized by nitric oxide insufficiency is a cardiovascular disease; a disease resulting from oxidative stress; low-renin hypertension; salt-sensitive hypertension; low-renin, salt-sensitive hypertension; primary pulmonary hypertension; thromboembolic pulmonary hypertension; pregnancy-induced hypertension; renovascular hypertension; hypertension-dependent end-stage renal disease; heart failure; microvascular cardiac ischemia; left ventricular hypertrophy with disproportionate microvascularization or diastolic dysfunction.

8. The method of claim 7, wherein the cardiovascular disease is congestive heart failure, hypertension, pulmonary hypertension, myocardial and cerebral infarctions, atherosclerosis, atherogenesis, thrombosis, ischemic heart disease, post-angioplasty restenosis, coronary artery diseases, renal failure, stable, unstable and variant (Prinzmetal) angina, cardiac edema, renal insufficiency, nephrotic edema, hepatic edema, stroke, transient ischemic attacks, cerebrovascular accidents, restenosis, controlling blood pressure in hypertension, platelet adhesion, platelet aggregation, smooth muscle cell proliferation, pulmonary edema, vascular complications associated with the use of medical devices, wounds associated with the use of medical devices, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia or bleeding disorders.

9. The method of claim 8, wherein the cardiovascular disease is congestive heart failure, hypertension, restenosis or atherosclerosis.

10. The method of claim 7, wherein the disease resulting from oxidative stress is atherogenesis, atheromatosis, arteriosclerosis, artherosclerosis, vascular hypertrophy associated with hypertension, hyperlipoproteinaemia, normal vascular degeneration through aging, parathyroidal reactive hyperplasia, chronic renal disease, a neoplastic disease, an inflammatory disease, a neurological and acute bronchopulmonary disease, a tumorigenesis, an ischemia-reperfusion syndrome, arthritis or sepsis.

11. The method of claim 6, wherein the composition is administered intravenously, orally, bucally, parenterally, by an inhalation spray, by topical application or transdermally.

12. A method of treating Raynaud's syndrome in a patient comprising administering to the patient a therapeutically effective amount of the composition of claim 5.

13. The method of claim 12, wherein the composition is administered orally or transdermally.

14. The method of claim 13, wherein the transdermal application is a sustained-release patch.

15. A composition comprising at least one compound of claim 3, or a stereoisomer thereof, or a pharmaceutically acceptable salt thereof, and at least one compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor or is a substrate for nitric oxide synthase or a pharmaceutically acceptable salt thereof.

16. The composition of claim 15, wherein the at least one compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor or is a substrate for nitric oxide synthase is an S-nitrosothiol.

17. The composition of claim 16, wherein the S-nitrosothiol is S-nitroso-N-acetylcysteine, S-nitroso-captopril, S-nitroso-N-acetylpenicillamine, S-nitroso-homocysteine, S-nitroso-cysteine, S-nitroso-glutathione or S-nitroso-cysteinyl-glycine.

18. The composition of claim 16, wherein the S-nitrosothiol is:

(i) $HS(C(R_e)(R_f))_mSNO$;

(ii) $ONS(C(R_e)(R_f))_mR_e$; and

(iii) $H_2N\text{-}CH(CO_2H)\text{-}(CH_2)_m\text{-}C(O)NH\text{-}CH(CH_2SNO)\text{-}C(O)NH\text{-}CH_2\text{-}CO_2H$;

wherein m is an integer from 2 to 20; R_e and R_f are each independently a hydrogen, an alkyl, a cycloalkoxy, a halogen, a hydroxy, an hydroxyalkyl, an alkoxyalkyl, an arylheterocyclic ring, an alkylaryl, an alkylcycloalkyl, an alkylheterocyclic ring, a cycloalkylalkyl, a cycloalkylthio, a cycloalkenyl, an heterocyclicalkyl, an alkoxy, a haloalkoxy, an amino, an alkylamino, a dialkylamino, an arylamino, a diarylamino, an alkylarylamino, an alkoxyhaloalkyl, a haloalkoxy, a sulfonic acid, a sulfonic ester, an alkylsulfonic acid, an arylsulfonic acid, an arylalkoxy, an alkylthio, an arylthio, a cyano an aminoalkyl, an aminoaryl, an aryl, an arylalkyl, an alkylaryl, a carboxamido, a alkylcarboxamido, an arylcarboxamido, an amidyl, a carboxyl, a carbamoyl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarbonyl, an arylcarbonyl, an ester, a carboxylic ester, an alkylcarboxylic ester, an arylcarboxylic ester, a haloalkoxy, a sulfonamido, an alkylsulfonamido, an arylsulfonamido, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfonyl, arylsulphonyloxy, a sulfonic ester, a urea, a phosphoryl, a nitro, W_h, -T-Q , or $-(C(R_e)(R_f))_k$-T-Q, or R_e and R_f taken together with the carbons to which they are attached form a carbonyl, a methanthial, a heterocyclic ring, a cycloalkyl group, an aryl group, an oxime or a bridged cycloalkyl group; Q is -NO or $-NO_2$; and T is independently a covalent bond, a carbonyl, an oxygen, $-S(O)_o$- or $-N(R_a)R_i$-; wherein o is an integer from 0 to 2; R_a is a lone pair of electrons, a hydrogen or an alkyl group; R_i is a hydrogen, an alkyl, an aryl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarboxylic ester, an arylcarboxylic ester, an alkylcarboxamido, an arylcarboxamido, an alkylaryl, an alkylsulfinyl, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfinyl, an arylsulfonyl, arylsulphonyloxy, a sulfonamido, a carboxamido, a carboxylic ester, an aminoalkyl, an aminoaryl, $-CH_2-C(T-Q)(R_e)(R_f)$, a bond to an adjacent atom creating a double bond to that atom, $-(N_2O_2-)^-\bullet M^+$, wherein M^+ is an organic or inorganic cation; with the proviso that when R_i is $-CH_2-C(T-Q)(R_e)(R_f)$ or $-(N_2O_2-)\bullet M^+$; then "-T-Q" can be a hydrogen, an alkyl group, an alkoxyalkyl group, an aminoalkyl group, a hydroxy group or an aryl group.

19. The composition of claim 15, wherein the at least one compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor, or is a substrate for nitric oxide synthase is:

(i) a compound that comprises at least one ON-O-, ON-N- or ON-C- group;

(ii) a compound that comprises at least one O_2N-O-, O_2N-N-, O_2N-S- or $-O_2N$-C- group;

(iii) a N-oxo-N-nitrosoamine having the formula: R^1R^2N-$N(O$-$M^+)$-NO, wherein R^1 and R^2 are each independently a polypeptide, an amino acid, a sugar, an oligonucleotide, a straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted hydrocarbon, or a heterocyclic group, and M^+ is an organic or inorganic cation.

20. The composition of claim 19, wherein the compound comprising at least one ON-O-, ON-N- or ON-C- group is an ON-O-polypeptide, an ON-N-polypepetide, an ON-C-polypeptide, an ON-O-amino acid, an ON-N-amino acid, an ON-C-amino acid, an ON-O-sugar, an ON-N-sugar, an ON-C-sugar, an ON-O-oligonucleotide, an ON-N-oligonucleotide, an ON-C-oligonucleotide, a straight or branched, saturated or unsaturated, substituted or unsubstituted, aliphatic or aromatic ON-O-hydrocarbon, a straight or branched, saturated or unsaturated, substituted or unsubstituted, aliphatic or aromatic ON-N-hydrocarbon, a straight or branched, saturated or unsaturated, substituted or unsubstituted, aliphatic or aromatic ON-C-hydrocarbon, an ON-O-heterocyclic compound, an ON-N-heterocyclic compound or a ON-C-heterocyclic compound.

21. The composition of claim 19, wherein compound comprising at least one O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- group is an O_2N-O-polypeptide, an O_2N-N-polypeptide, an O_2N-S-polypeptide, an O_2N-C-polypeptide, an O_2N-O-amino acid, O_2N-N-amino acid, O_2N-S-amino acid, an O_2N-C-amino acid, an O_2N-O-sugar, an O_2N-N-sugar, O_2N-S-sugar, an O_2N-C-sugar, an O_2N-O-oligonucleotide, an O_2N-N-oligonucleotide, an O_2N-S-oligonucleotide, an O_2N-C-oligonucleotide, a straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted O_2N-O-hydrocarbon, a straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted O_2N-N-hydrocarbon, a straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted O_2N-S-hydrocarbon, a straight or branched, saturated or unsaturated, aliphatic or aromatic, substituted or unsubstituted O_2N-C-hydrocarbon, an O_2N-O-heterocyclic compound, an O_2N-N-heterocyclic compound, an O_2N-S-heterocyclic compound or an O_2N-C-heterocyclic compound.

22. The composition of claim 21, wherein compound comprising at least one O_2N-O-, O_2N-N-, O_2N-S- or O_2N-C- group is isosorbide mononitrate and/or isosorbide dinitrate.

23. The composition of claim 15, wherein the at least one compound that

donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor, or is a substrate for nitric oxide synthase, is L-arginine, L-homoarginine, N-hydroxy-L-arginine, nitrosated L-arginine, nitrosylated L-arginine, nitrosated N-hydroxy-L-arginine, nitrosylated N-hydroxy-L-arginine, citrulline, ornithine, glutamine, lysine, polypeptides comprising at least one of these amino acids or inhibitors of the enzyme arginase.

24. A method of treating and/or preventing a vascular disease characterized by nitric oxide insufficiency in a patient in need thereof comprising administering a therapeutically effective amount of the composition of claim 15.

25. The method of claim 24, wherein the vascular disease characterized by nitric oxide insufficiency is a cardiovascular disease; a disease resulting from oxidative stress; low-renin hypertension; salt-sensitive hypertension; low-renin, salt-sensitive hypertension; primary pulmonary hypertension; thromboembolic pulmonary hypertension; pregnancy-induced hypertension; renovascular hypertension; hypertension-dependent end-stage renal disease; heart failure; microvascular cardiac ischemia; left ventricular hypertrophy with disproportionate microvascularization or diastolic dysfunction.

26. The method of claim 25, wherein the cardiovascular disease is congestive heart failure, hypertension, pulmonary hypertension, myocardial and cerebral infarctions, atherosclerosis, atherogenesis, thrombosis, ischemic heart disease, post-angioplasty restenosis, coronary artery diseases, renal failure, stable, unstable and variant (Prinzmetal) angina, cardiac edema, renal insufficiency, nephrotic edema, hepatic edema, stroke, transient ischemic attacks, cerebrovascular accidents, restenosis, controlling blood pressure in hypertension, platelet adhesion, platelet aggregation, smooth muscle cell proliferation, pulmonary edema, vascular complications associated with the use of medical devices, wounds associated with the use of medical devices, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia or bleeding disorders.

27. The method of claim 26, wherein the cardiovascular disease is congestive heart failure, hypertension, restenosis or atherosclerosis.

28. The method of claim 25, wherein the disease resulting from oxidative stress is atherogenesis, atheromatosis, arteriosclerosis, artherosclerosis, vascular hypertrophy associated with hypertension, hyperlipoproteinaemia, normal vascular degeneration through aging, parathyroidal reactive hyperplasia, chronic renal disease, a neoplastic disease, an inflammatory disease, a neurological and acute bronchopulmonary disease, a

tumorigenesis, an ischemia-reperfusion syndrome, arthritis or sepsis.

29. The method of claim 24, wherein the composition is administered intravenously, orally, bucally, parenterally, by an inhalation spray, by topical application or transdermally.

30. A method of treating Raynaud's syndrome in a patient comprising administering to the patient a therapeutically effective amount of the composition of claim 15.

31. The method of claim 30, wherein the composition is administered orally or transdermally.

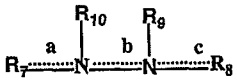
32. The method of claim 31, wherein the transdermal application is a sustained-release patch.

33. The composition of claim 3, further comprising at least one antioxidant.

34. The composition of claim 33, wherein the antioxidant is a small-molecule antioxidant, or a pharmaceutically acceptable salt thereof, or an antioxidant enzyme.

35. The composition of claim 34, wherein the small-molecule antioxidant is a hydralazine compound of Formula (VI), a glutathione, a vitamin C, a vitamin E, a cysteine, a N-acetyl-cysteine, a β-carotene, an ubiquinone, an ubiquinol-10, a tocopherol, a coenzyme Q, or a mixture thereof;

wherein the hydralazine compound of Formula (VI) is:



(VI)

wherein a, b and c are independently a single or double bond; R_7 and R_8 are each independently a hydrogen, an alkyl, an ester or a heterocyclic ring; R_9 and R_{10} are each independently a lone pair of electrons or a hydrogen; with the proviso that at least one of R_7, R_8, R_9 and R_{10} is not a hydrogen.

36. The composition of claim 34, wherein the antioxidant enzyme is a superoxide dismutase, a catalase, a glutathione peroxidase, or a mixture thereof.

37. The composition of claim 35, wherein the hydralazine compound is budralazine, cadralazine, dihydralazine, endralazine, hydralazine, pildralazine or todralazine or a pharmaceutically acceptable salt thereof.

38. The composition of claim 37, wherein the hydralazine compound is

hydralazine hydrochloride.

39. A method of treating and/or preventing a vascular disease characterized by nitric oxide insufficiency in a patient in need thereof comprising administering a therapeutically effective amount of the composition of claim 33.

40. The method of claim 39, wherein the vascular disease characterized by nitric oxide insufficiency is a cardiovascular disease; a disease resulting from oxidative stress; low-renin hypertension; salt-sensitive hypertension; low-renin, salt-sensitive hypertension; primary pulmonary hypertension; thromboembolic pulmonary hypertension; pregnancy-induced hypertension; renovascular hypertension; hypertension-dependent end-stage renal disease; heart failure; microvascular cardiac ischemia; left ventricular hypertrophy with disproportionate microvascularization or diastolic dysfunction.

41. The method of claim 40, wherein the cardiovascular disease is congestive heart failure, hypertension, pulmonary hypertension, myocardial and cerebral infarctions, atherosclerosis, atherogenesis, thrombosis, ischemic heart disease, post-angioplasty restenosis, coronary artery diseases, renal failure, stable, unstable and variant (Prinzmetal) angina, cardiac edema, renal insufficiency, nephrotic edema, hepatic edema, stroke, transient ischemic attacks, cerebrovascular accidents, restenosis, controlling blood pressure in hypertension, platelet adhesion, platelet aggregation, smooth muscle cell proliferation, pulmonary edema, vascular complications associated with the use of medical devices, wounds associated with the use of medical devices, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia or bleeding disorders.

42. The method of claim 41, wherein the cardiovascular disease is congestive heart failure, hypertension, restenosis or atherosclerosis.

43. The method of claim 40, wherein the disease resulting from oxidative stress is atherogenesis, atheromatosis, arteriosclerosis, artherosclerosis, vascular hypertrophy associated with hypertension, hyperlipoproteinaemia, normal vascular degeneration through aging, parathyroidal reactive hyperplasia, chronic renal disease, a neoplastic disease, an inflammatory disease, a neurological and acute bronchopulmonary disease, a tumorigenesis, an ischemia-reperfusion syndrome, arthritis or sepsis.

44. The method of claim 39, wherein the composition is administered intravenously, orally, bucally, parenterally, by an inhalation spray, by topical application or transdermally.

45. A method of treating Raynaud's syndrome in a patient comprising

administering to the patient a therapeutically effective amount of the composition of claim 33.

46. The method of claim 45, wherein the composition is administered orally or transdermally.

47. The method of claim 46, wherein the transdermal application is a sustained-release patch.

48. The composition of claim 3, further comprising at least one nitrosated compound used to treat cardiovascular diseases.

49. The composition of claim 48, wherein the nitrosated compound used to treat cardiovascular diseases is a nitrosated angiotensin-converting enzyme inhibitor, a nitrosated beta-adrenergic blocker, a nitrosated cholesterol reducer, a nitrosated calcium channel blocker, a nitrosated endothelin antagonist, a nitrosated angiotensin II receptor antagonist, a nitrosated renin inhibitor, or a mixture thereof.

50. A method of treating and/or preventing a vascular disease characterized by nitric oxide insufficiency in a patient in need thereof comprising administering a therapeutically effective amount of the composition of claim 48.

51. The method of claim 50, wherein the vascular disease characterized by nitric oxide insufficiency is a cardiovascular disease; a disease resulting from oxidative stress; low-renin hypertension; salt-sensitive hypertension; low-renin, salt-sensitive hypertension; primary pulmonary hypertension; thromboembolic pulmonary hypertension; pregnancy-induced hypertension; renovascular hypertension; hypertension-dependent end-stage renal disease; heart failure; microvascular cardiac ischemia; left ventricular hypertrophy with disproportionate microvascularization or diastolic dysfunction.

52. The method of claim 51, wherein the cardiovascular disease is congestive heart failure, hypertension, pulmonary hypertension, myocardial and cerebral infarctions, atherosclerosis, atherogenesis, thrombosis, ischemic heart disease, post-angioplasty restenosis, coronary artery diseases, renal failure, stable, unstable and variant (Prinzmetal) angina, cardiac edema, renal insufficiency, nephrotic edema, hepatic edema, stroke, transient ischemic attacks, cerebrovascular accidents, restenosis, controlling blood pressure in hypertension, platelet adhesion, platelet aggregation, smooth muscle cell proliferation, pulmonary edema, vascular complications associated with the use of medical devices, wounds associated with the use of medical devices, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia or bleeding disorders.

53. The method of claim 52, wherein the cardiovascular disease is congestive heart failure, hypertension, restenosis or atherosclerosis.

54. The method of claim 51, wherein the disease resulting from oxidative stress is atherogenesis, atheromatosis, arteriosclerosis, artherosclerosis, vascular hypertrophy associated with hypertension, hyperlipoproteinaemia, normal vascular degeneration through aging, parathyroidal reactive hyperplasia, chronic renal disease, a neoplastic disease, an inflammatory disease, a neurological and acute bronchopulmonary disease, a tumorigenesis, an ischemia-reperfusion syndrome, arthritis or sepsis.

55. The method of claim 50, wherein the composition is administered intravenously, orally, bucally, parenterally, by an inhalation spray, by topical application or transdermally.

56. A method of treating Raynaud's syndrome in a patient comprising administering to the patient a therapeutically effective amount of the composition of claim 48.

57. The method of claim 56, wherein the composition is administered orally or transdermally.

58. The method of claim 57, wherein the transdermal application is a sustained-release patch.

59. The composition of claim 3, further comprising at least one compound used to treat cardiovascular diseases, or a pharmaceutically acceptable salt thereof.

60. The composition of claim 59, wherein the at least one compound used to treat cardiovascular diseases is an angiotensin-converting enzyme inhibitor, a beta-adrenergic blocker, a cholesterol reducer, a calcium channel blocker, an angiotensin II receptor antagonist, an endothelin antagonist, a renin inhibitor, or a mixture thereof.

61. A method of treating and/or preventing a vascular disease characterized by nitric oxide insufficiency in a patient in need thereof comprising administering a therapeutically effective amount of the composition of claim 59.

62. The method of claim 61, wherein the vascular disease characterized by nitric oxide insufficiency is a cardiovascular disease; a disease resulting from oxidative stress; low-renin hypertension; salt-sensitive hypertension; low-renin, salt-sensitive hypertension; primary pulmonary hypertension; thromboembolic pulmonary hypertension; pregnancy-induced hypertension; renovascular hypertension; hypertension-dependent end-stage renal disease; heart failure; microvascular cardiac ischemia; left ventricular

hypertrophy with disproportionate microvascularization or diastolic dysfunction.

63. The method of claim 62, wherein the cardiovascular disease is congestive heart failure, hypertension, pulmonary hypertension, myocardial and cerebral infarctions, atherosclerosis, atherogenesis, thrombosis, ischemic heart disease, post-angioplasty restenosis, coronary artery diseases, renal failure, stable, unstable and variant (Prinzmetal) angina, cardiac edema, renal insufficiency, nephrotic edema, hepatic edema, stroke, transient ischemic attacks, cerebrovascular accidents, restenosis, controlling blood pressure in hypertension, platelet adhesion, platelet aggregation, smooth muscle cell proliferation, pulmonary edema, vascular complications associated with the use of medical devices, wounds associated with the use of medical devices, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia or bleeding disorders.

64. The method of claim 63, wherein the cardiovascular disease or disorder is congestive heart failure, hypertension, restenosis or atherosclerosis.

65. The method of claim 62, wherein the disease resulting from oxidative stress is atherogenesis, atheromatosis, arteriosclerosis, artherosclerosis, vascular hypertrophy associated with hypertension, hyperlipoproteinaemia, normal vascular degeneration through aging, parathyroidal reactive hyperplasia, chronic renal disease, a neoplastic disease, an inflammatory disease, a neurological and acute bronchopulmonary disease, a tumorigenesis, an ischemia-reperfusion syndrome, arthritis or sepsis.

66. The method of claim 61, wherein the composition is administered intravenously, orally, bucally, parenterally, by an inhalation spray, by topical application or transdermally.

67. The method of claim 61, further comprising administering a digitalis.

68. The method of claim 67, wherein the digitalis is digoxin

69. The method of claim 67, wherein the digoxin is administered in an amount to achieve a blood serum concentration of at least about 0.7 nanograms per milliliter to about 2.0 nanograms per milliliter.

70. The method of claim 61 further comprising administering a therapeutically effective edema managing amount of a diuretic compound.

71. The method of claim 70, wherein the diuretic compound is a thiazide, ethacrynic acid, a furosemide, a spiranolactone, a triamterene, or a mixture thereof.

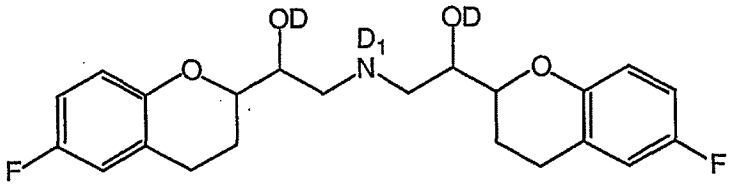
72. The method of claim 70, further comprising administering a therapeutically effective amount of potassium.

73. The method of claim 72, wherein the potassium is administered as potassium chloride or by the daily ingestion of foods with high potassium content.

74. A composition comprising at least one compound of Formula (I), Formula (II), Formula (III), Formula (IV) or Formula (V), or an isomer thereof, or a pharmaceutically acceptable salt thereof, bound to a matrix;

wherein the matrix is a polymer, a fiber, or a mixture thereof; and

wherein the compound of Formula (I) is:

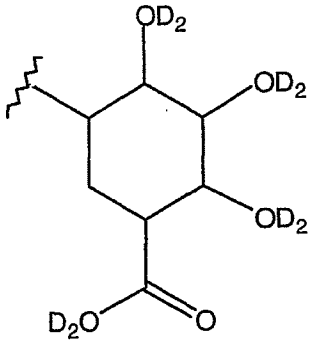


I

wherein:

D is hydrogen, Q, K or R_5;

R_5 is:



D_1 is hydrogen or R_5;

D_2 is hydrogen, Q or K;

Q is –NO or $-NO_2$;

K is $-W_a-E_b-(C(R_e)(R_f))_p-E_c-(C(R_e)(R_f))_x-W_d-(C(R_e)(R_f))_y-W_i-E_j-W_g-(C(R_e)(R_f))_z-$ T-Q;

a, b, c, d, g, i and j are each independently an integer from 0 to 3;

p, x, y and z are each independently an integer from 0 to 10;

W at each occurrence is independently -C(O)-, -C(S)-, -T-, $-(C(R_e)(R_f))_h-$, an alkyl group, an aryl group, a heterocyclic ring, an arylheterocyclic ring, or $-(CH_2CH_2O)_q-$;

E at each occurrence is independently -T-, an alkyl group, an aryl group, $-(C(R_e)(R_f))_h-$, a heterocyclic ring, an arylheterocyclic ring, or $-(CH_2CH_2O)_q-$;

h is an integer form 1 to 10;

q is an integer from 1 to 5;

R_e and R_f are each independently a hydrogen, an alkyl, a cycloalkoxy, a halogen, a hydroxy, an hydroxyalkyl, an alkoxyalkyl, an arylheterocyclic ring, an alkylaryl, an alkylcycloalkyl, an alkylheterocyclic ring, a cycloalkylalkyl, a cycloalkylthio, a cycloalkenyl, an heterocyclicalkyl, an alkoxy, a haloalkoxy, an amino, an alkylamino, a dialkylamino, an arylamino, a diarylamino, an alkylarylamino, an alkoxyhaloalkyl, a haloalkoxy, a sulfonic acid, a sulfonic ester, an alkylsulfonic acid, an arylsulfonic acid, an arylalkoxy, an alkylthio, an arylthio, a cyano an aminoalkyl, an aminoaryl, an aryl, an arylalkyl, an alkylaryl, a carboxamido, a alkylcarboxamido, an arylcarboxamido, an amidyl, a carboxyl, a carbamoyl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarbonyl, an arylcarbonyl, an ester, a carboxylic ester, an alkylcarboxylic ester, an arylcarboxylic ester, a haloalkoxy, a sulfonamido, an alkylsulfonamido, an arylsulfonamido, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfonyl, arylsulphonyloxy, a sulfonic ester, a urea, a phosphoryl, a nitro, W_h, -T-Q , or $-(C(R_e)(R_f))_k$-T-Q, or R_e and R_f taken together with the carbons to which they are attached form a carbonyl, a methanthial, a heterocyclic ring, a cycloalkyl group, an aryl group, an oxime or a bridged cycloalkyl group;

k is an integer from 1 to 3;

T at each occurrence is independently a covalent bond, a carbonyl, an oxygen, $-S(O)_o-$ or $-N(R_a)R_i-$;

o is an integer from 0 to 2;

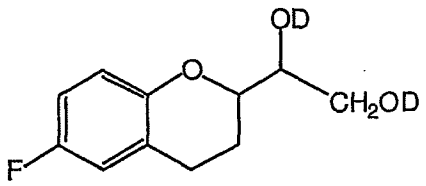
R_a is a lone pair of electrons, a hydrogen or an alkyl group;

R_i is a hydrogen, an alkyl, an aryl, an alkylcarboxylic acid, an arylcarboxylic acid, an alkylcarboxylic ester, an arylcarboxylic ester, an alkylcarboxamido, an arylcarboxamido, an alkylaryl, an alkylsulfinyl, an alkylsulfonyl, an alkylsulfonyloxy, an arylsulfinyl, an arylsulfonyl, arylsulphonyloxy, a sulfonamido, a carboxamido, a carboxylic ester, an aminoalkyl, an aminoaryl, $-CH_2-C(T-Q)(R_e)(R_f)$, a bond to an adjacent atom creating a double bond to that atom,

$-(N_2O_2-)^{-}\bullet M^{+}$, wherein M^{+} is an organic or inorganic cation;

with the proviso that the compound of Formula (I) must contain at least one nitrite, nitrate, thionitrite or thionitrate group;

wherein the compounds of Formula (II), Formula (III), Formula (IV) and Formula (V) are:



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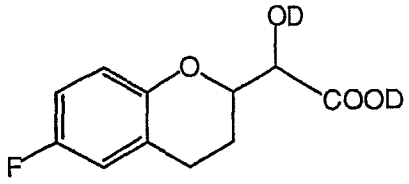
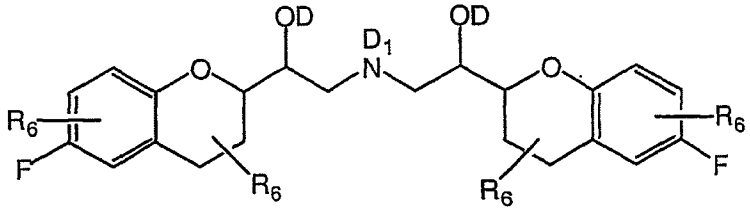
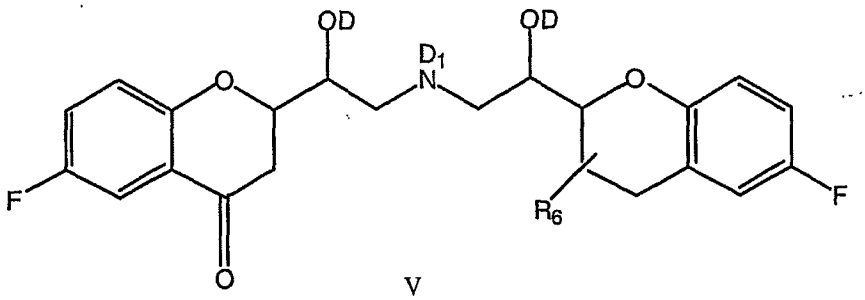


III



IV



V

wherein:

R_6 at each occurrence is independently a hydrogen, a hydroxy or -OD;

D and $D_1$is as defined herein; and

with the proviso that the compounds of Formula (II), Formula (III), Formula (IV) and

Formula (V), must contain at least one nitrite, nitrate, thionitrite or thionitrate group.

75. The composition of claim 74, wherein the polymer is a synthetic polymer or a natural polymer selected from a polyolefin, a polyethylenimine, a polyethyleneimine derivative, a polyether, a polyanhydride, a polyhydroxybutyrate, a polyester, a polyamide, a polyurethane, a biopolymer, a starburst dendrimer, or a mixture thereof.

76. The composition of claim 74, further comprising at least one compound that donates, transfers or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor, or is a substrate for nitric oxide synthase, or at least one therapeutic agent or a mixture thereof.

77. The composition of claim 76, wherein the therapeutic agent is an antithrombogenic agent, a thrombolytic agent, a fibrinolytic agent, a vasospasm inhibitor, a potassium channel activator, a calcium channel blocker, an antihypertensive agent, an antimicrobial agent, an antibiotic, an antiplatelet agent, an antimitotic agent, an antiproliferative agent, a microtubule inhibitor, an antisecretory agent, a remodelling inhibitor, an antisense nucleotide, an anti-cancer chemotherapeutic agent, a steroid, a non-steroidal antiinflammatory agent, a selective COX-2 inhibitor, an immunosuppressive agent, a growth factor antagonist or antibody, a dopamine agonist, a radiotherapeutic agent, a heavy metal functioning as a radiopaque agent, a biologic agent, an angiotensin converting enzyme inhibitor, an angiotensin II receptor antagonist, a renin inhbitior, a free radical scavenger, an iron chelator, an antioxidant, a sex hormone, an antipolymerase, an antiviral agent, a photodynamic therapy agent, an antibody targeted therapy agent, a gene therapy agent, or a mixture thereof.

78. A method for direct delivery of nitric oxide to a targeted site in a patient in need thereof comprising administering the composition of claim 74 or 76 directly to the targeted site in the patient.

79. The method of claim 78, wherein the composition provides sustained delivery of nitric oxide to the targeted sited in the patient.

80. A medical device comprising the composition of claim 74 or 76.

81. The medical device of claim 79, wherein the composition coats all or a portion of the surface of the medical device.

82. The medical device of claim 80, wherein the composition forms all or part of the medical device.

83. The medical device of claim 80, wherein the medical device is a balloon, a

catheter tip, a stent, a catheter, a prosthetic heart valve, a synthetic vessel graft, an arteriovenous shunt, a heart valve, a suture, a vascular implant, a drug pump, a drug delivery catheter, plastic tubing, a dialysis bag, a lead, a pacemaker, an implantable pulse generator, an implantable cardiac defibrillator, a cardioverter defibrillator, a defibrillator, a spinal stimulator, a brain stimulator, a sacral nerve stimulator, a chemical sensor or a membrane surface.

84. A method for the prevention of platelet aggregation and platelet adhesion caused by the exposure of blood to a medical device comprising incorporating at least one composition of claim 74 or 76 or a pharmaceutically acceptable salt thereof, into or on the medical device.

85. The method of claim 84, wherein the medical device is a balloon, a catheter tip, a stent, a catheter, a prosthetic heart valve, a synthetic vessel graft, an arteriovenous shunt, a heart valve, a suture, a vascular implant, a drug pump, a drug delivery catheter, plastic tubing, a dialysis bag, a lead, a pacemaker, an implantable pulse generator, an implantable cardiac defibrillator, a cardioverter defibrillator, a defibrillator, a spinal stimulator, a brain stimulator, a sacral nerve stimulator, a chemical sensor or a membrane surface.

86. The method of claim 84, wherein the blood is a blood product or a blood component.

87. A method for treating injured tissue in a patient in need thereof comprising administering at least one composition of claim 74 or 76 or a pharmaceutically acceptable salt thereof, to the site of the injured tissue in the patient.

88. The method of claim 87, wherein the injured tissue is a blood vessel.

89. The method of claim 87, wherein the compound is administered to the site of the injured tissue via at least one of a suture, a vascular implant, a stent, a heart valve, a drug pump or a drug delivery catheter.

90. A kit comprising at least one compound of claim 3 and at least one compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor or is a substrate for nitric oxide synthase, or a pharmaceutically acceptable salt thereof.

91. The kit of claim 90, further comprising at least one antioxidant and/or at least one compound used to treat cardiovascular diseases.

92. The kit of claim 90, wherein the compound of claim 3 and the compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor, or is a substrate for nitric oxide synthase are separate components in the kit or as a composition in the kit.

93. A composition comprising nebivolol and/or at least one metabolite of nebivolol, or a steroisomer thereof, or a pharmaceutically acceptable salt thereof and at least one compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor or is a substrate for nitric oxide synthase or a pharmaceutically acceptable salt thereof.

94. The composition of claim 93, wherein the at least one compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor or is a substrate for nitric oxide synthase is isosorbide mononitrate or isosorbide dinitrate.

95. The compositions of claim 93, further comprising at least one antioxidant, and/or at least one compound used to treat cardiovascular diseases.

96. The composition of claim 95, wherein the antioxidant is hydralazine hydrochloride.

97. A method of treating and/or preventing a vascular disease characterized by nitric oxide insufficiency in a patient in need thereof comprising administering a therapeutically effective amount of the composition of claim 93.

98. The method of claim 97, wherein the vascular disease characterized by nitric oxide insufficiency is a cardiovascular disease; a disease resulting from oxidative stress; low-renin hypertension; salt-sensitive hypertension; low-renin, salt-sensitive hypertension; primary pulmonary hypertension; thromboembolic pulmonary hypertension; pregnancy-induced hypertension; renovascular hypertension; hypertension-dependent end-stage renal disease; heart failure; microvascular cardiac ischemia; left ventricular hypertrophy with disproportionate microvascularization or diastolic dysfunction.

99. The method of claim 98, wherein the cardiovascular disease is congestive heart failure, hypertension, pulmonary hypertension, myocardial and cerebral infarctions, atherosclerosis, atherogenesis, thrombosis, ischemic heart disease, post-angioplasty restenosis, coronary artery diseases, renal failure, stable, unstable and variant (Prinzmetal) angina, cardiac edema, renal insufficiency, nephrotic edema, hepatic edema, stroke, transient ischemic attacks, cerebrovascular accidents, restenosis, controlling blood pressure in

hypertension, platelet adhesion, platelet aggregation, smooth muscle cell proliferation, pulmonary edema, vascular complications associated with the use of medical devices, wounds associated with the use of medical devices, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, thrombocytopenia or bleeding disorders.

100. The method of claim 99, wherein the cardiovascular disease is congestive heart failure, hypertension, restenosis or atherosclerosis.

101. The method of claim 98, wherein the disease resulting from oxidative stress is atherogenesis, atheromatosis, arteriosclerosis, artherosclerosis, vascular hypertrophy associated with hypertension, hyperlipoproteinaemia, normal vascular degeneration through aging, parathyroidal reactive hyperplasia, chronic renal disease, a neoplastic disease, an inflammatory disease, a neurological and acute bronchopulmonary disease, a tumorigenesis, an ischemia-reperfusion syndrome, arthritis or sepsis.

102. The method of claim 97, wherein the composition is administered intravenously, orally, bucally, parenterally, by an inhalation spray, by topical application or transdermally.

103. A method of treating Raynaud's syndrome in a patient comprising administering to the patient a therapeutically effective amount of the composition of claim 93.

104. The method of claim 103, wherein the composition is administered orally or transdermally.

105. The method of claim 104, wherein the transdermal application is a sustained-release patch.

106. A kit comprising nebivolol and/or at least one metabolite of nebivolol, or a stereoisomer thereof, and at least one compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived relaxing factor or is a substrate for nitric oxide synthase, or a pharmaceutically acceptable salt thereof.

107. The kit of claim 106, further comprising at least one antioxidant and/or at least one compound used to treat cardiovascular diseases.

108. The kit of claim 107, wherein the nebivolol and/or the at least one metabolite of nebivolol, or a stereoisomer thereof, and the compound that donates, transfers, or releases nitric oxide, or induces the production of endogenous nitric oxide or endothelium-derived

relaxing factor, or is a substrate for nitric oxide synthase are separate components in the kit or as a composition in the kit.

Figure 1

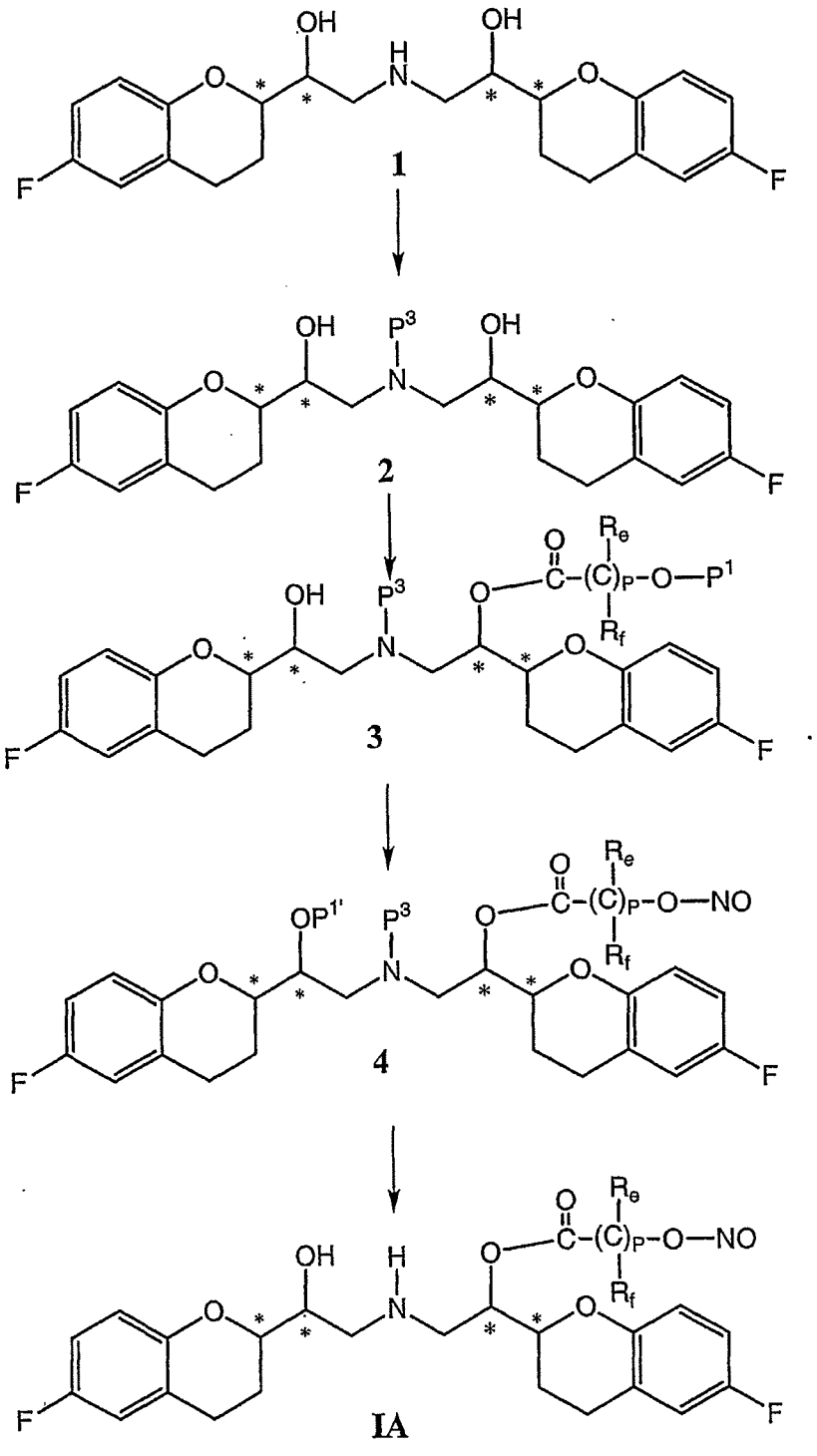


Figure 2

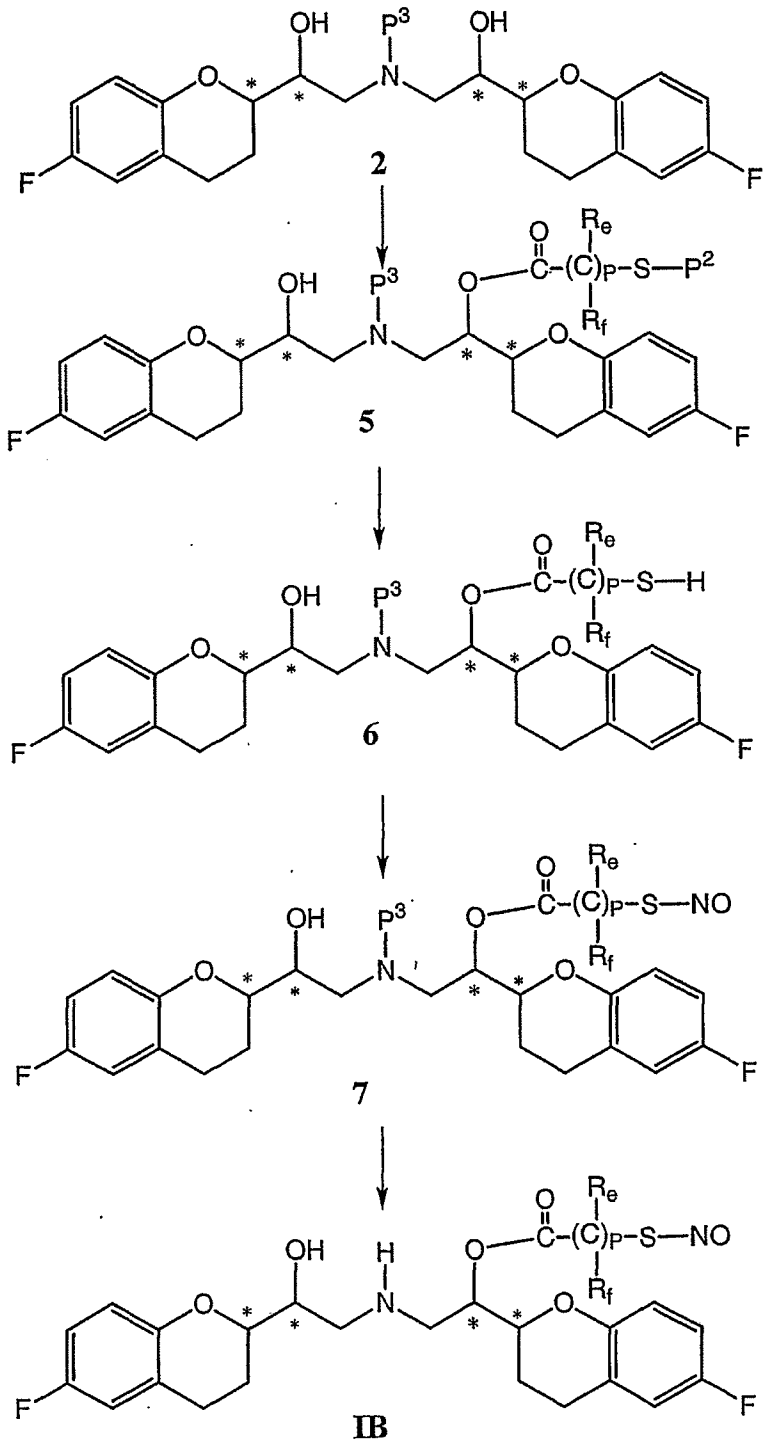


Figure 3

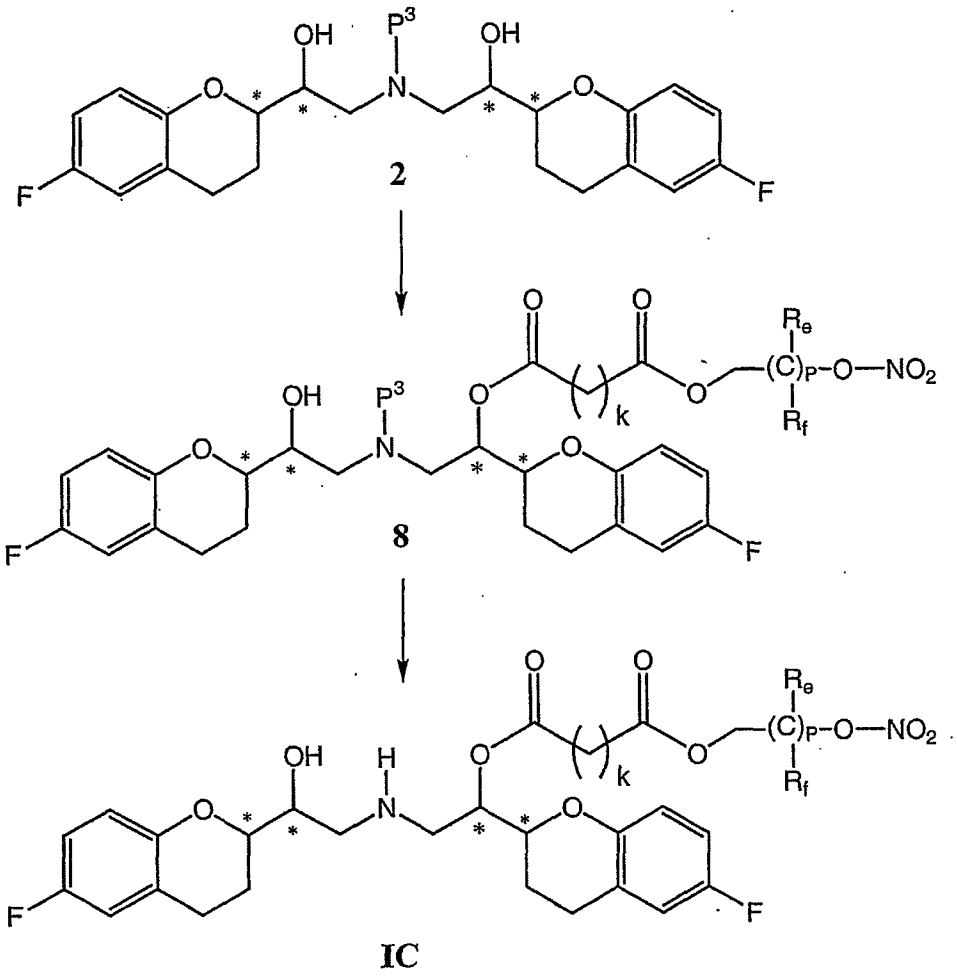


Figure 4

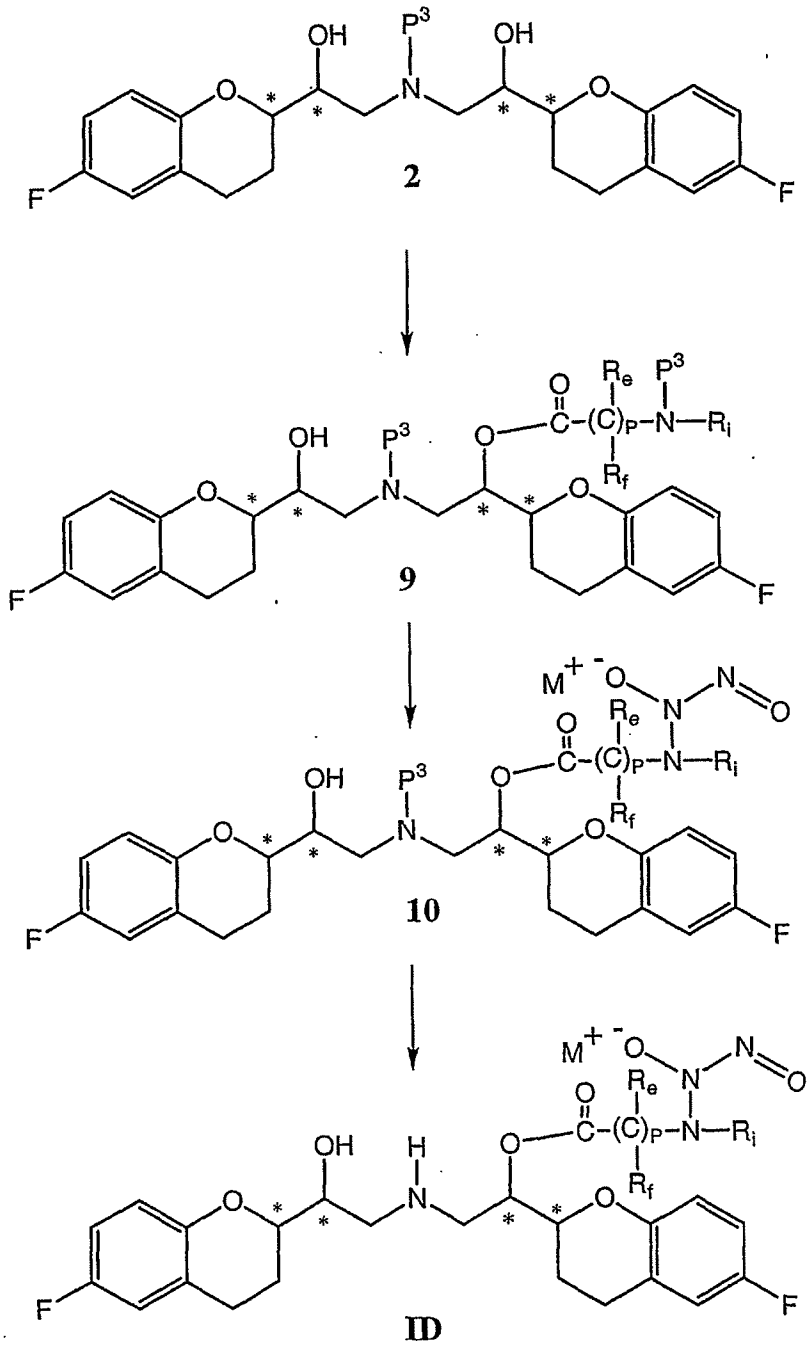
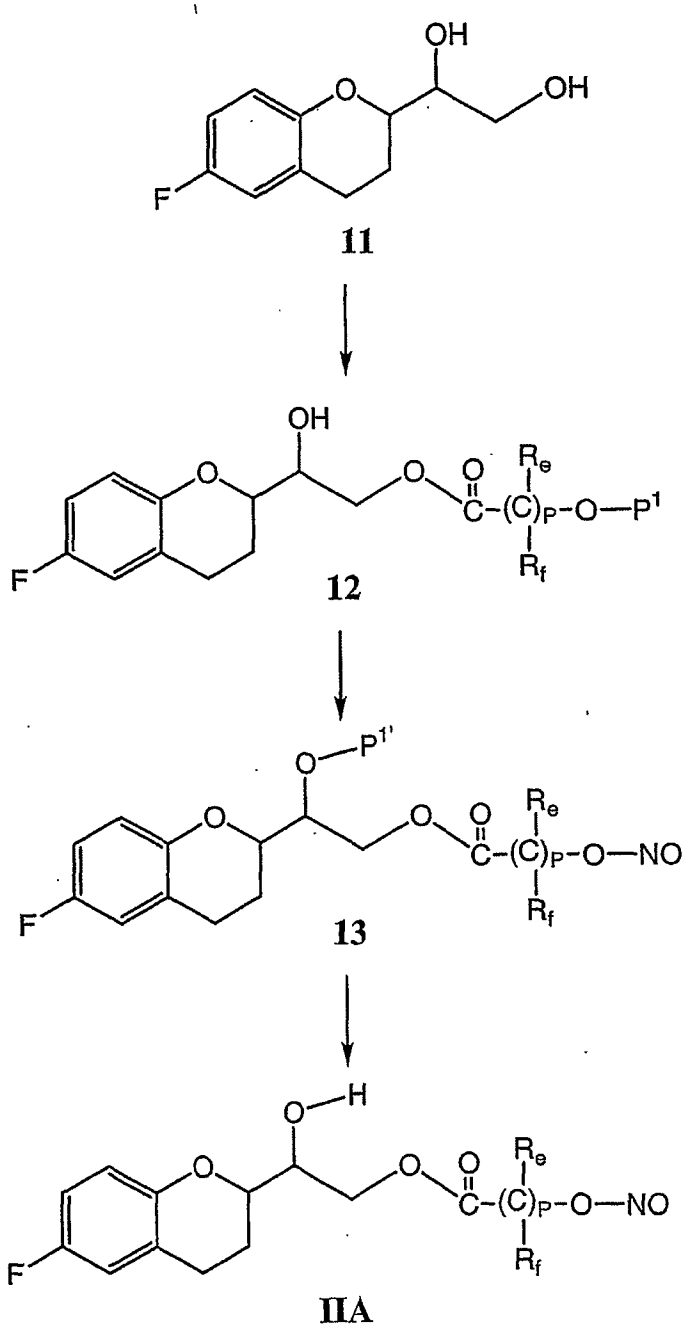


Figure 5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O
OH
F
11
OH
O
O
O
C-(C)P-O—P1
Re
Rf
F
12
O—P1'
O
O
O
C-(C)P-O—NO
Re
Rf
F
13
O—H
O
O
O
C-(C)P-O—NO
Re
Rf
F
IIA

Figure 6

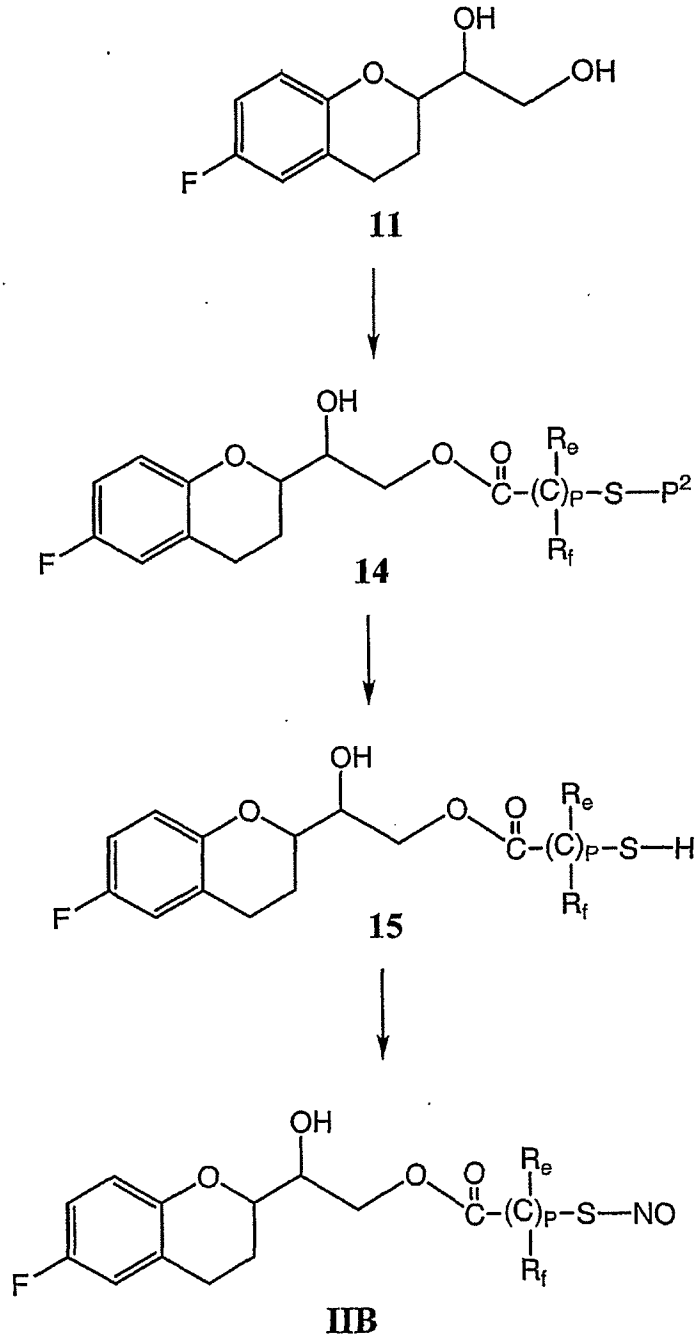
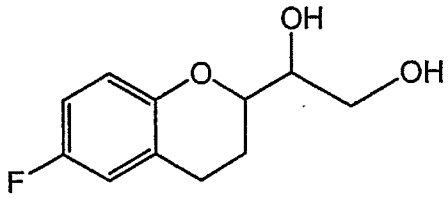

OH
O
OH
F
11
OH
O
O
O
Re
C-(C)P-S—P2
Rf
F
14
OH
O
O
O
Re
C-(C)P-S—H
Rf
F
15
OH
O
O
O
Re
C-(C)P-S—NO
Rf
F
IIB

Figure 7



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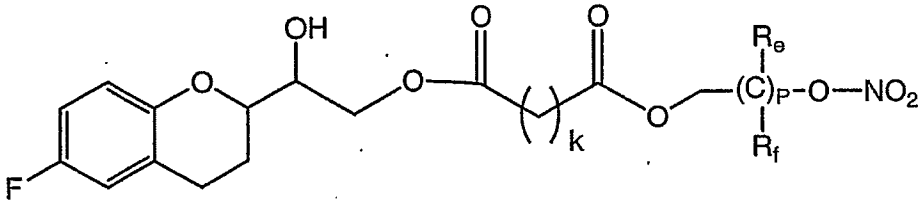

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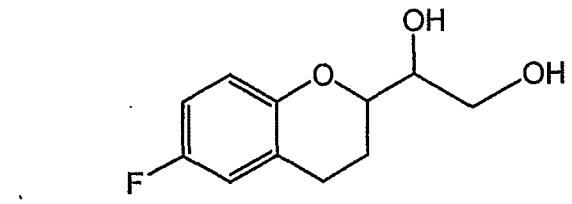
Figure 8



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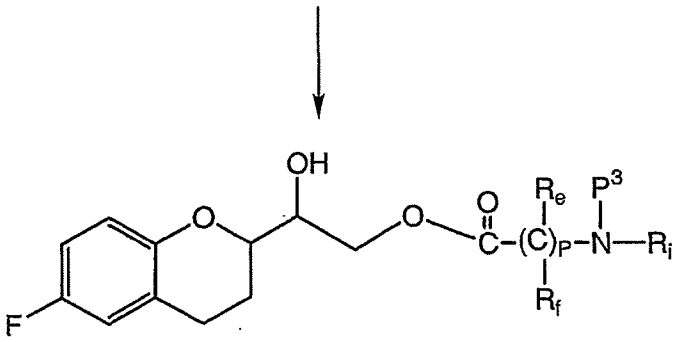

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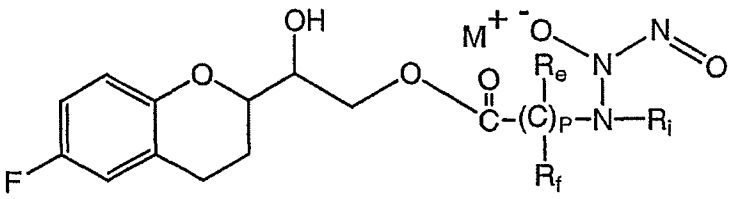

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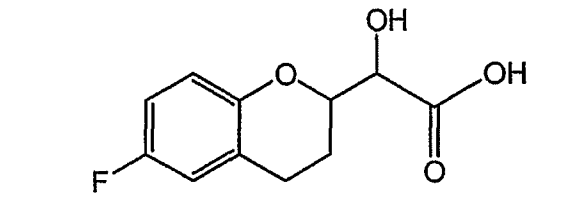
Figure 9



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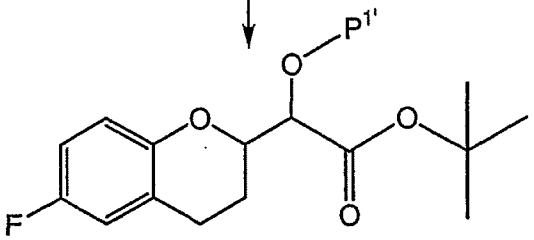

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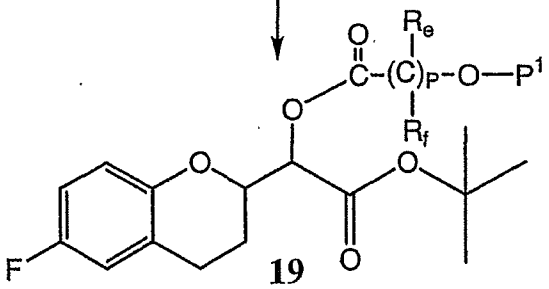

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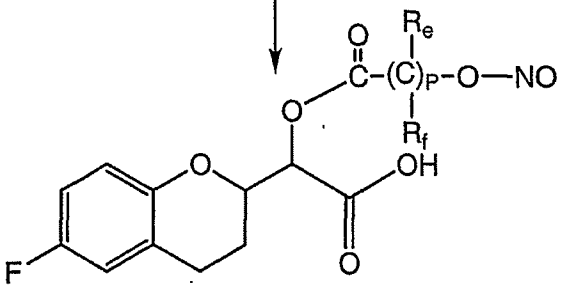

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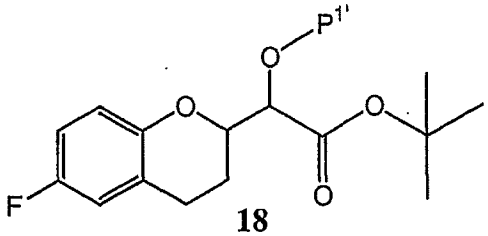
Figure 10



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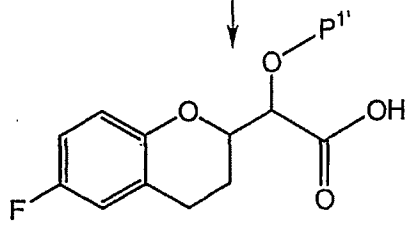

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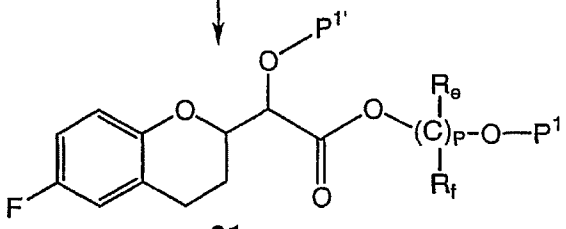

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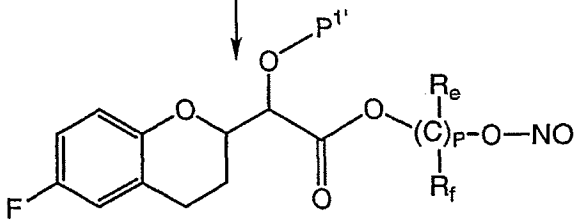

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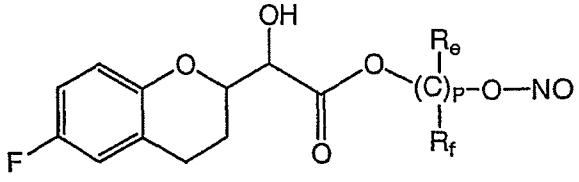
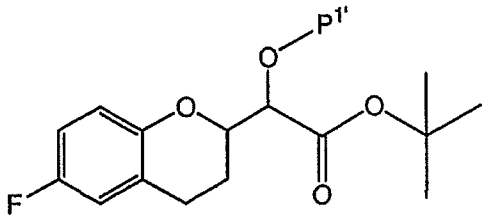


IIIB

Figure 11



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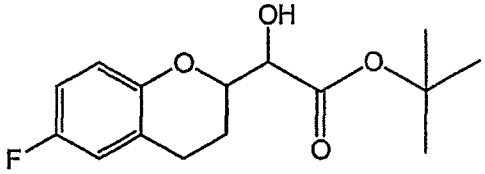

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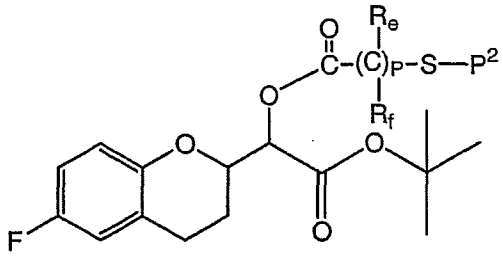

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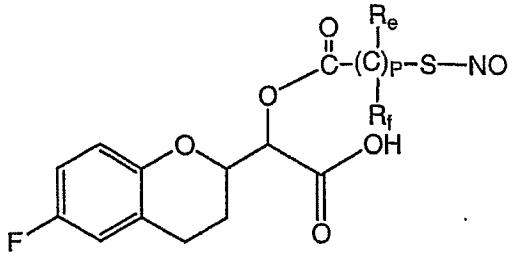

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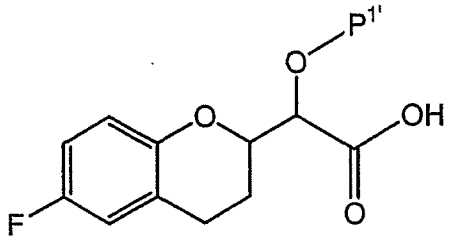
Figure 12



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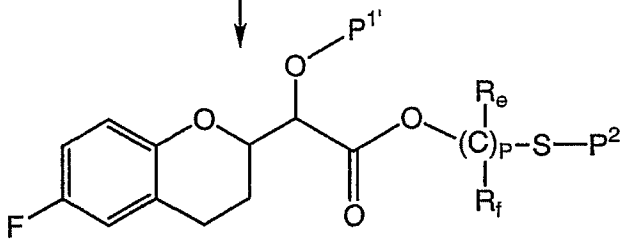

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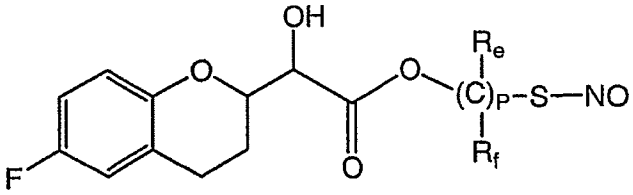

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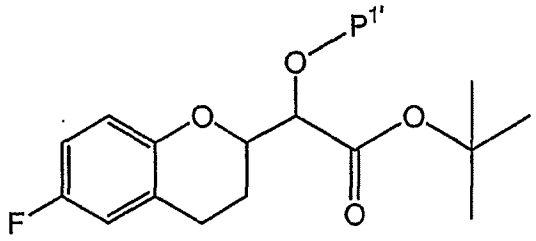
Figure 13



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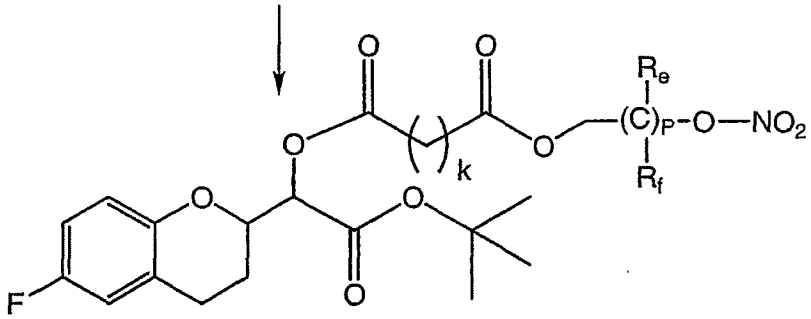

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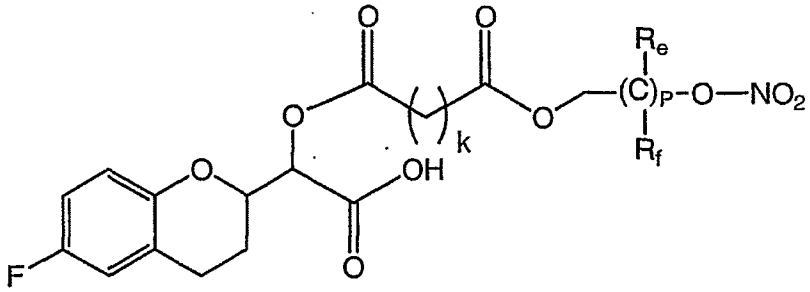

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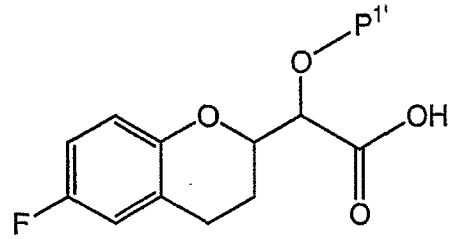
Figure 14



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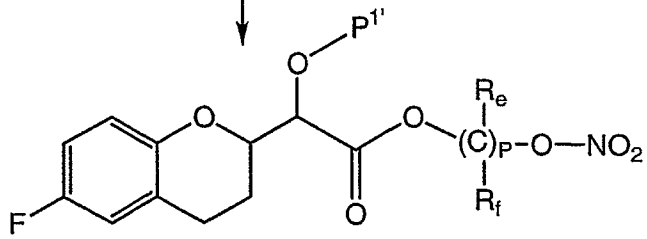

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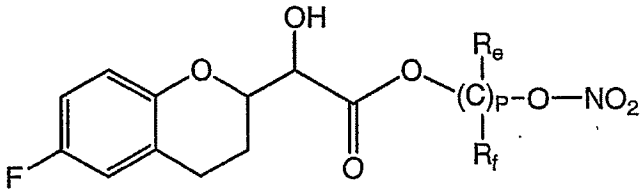

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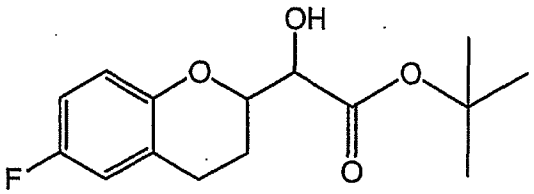
Figure 15



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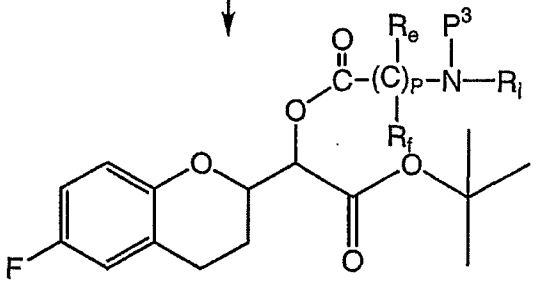

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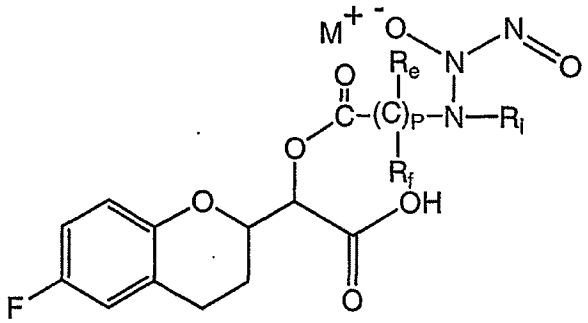
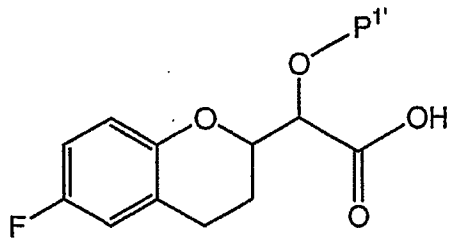


IIIG

Figure 16



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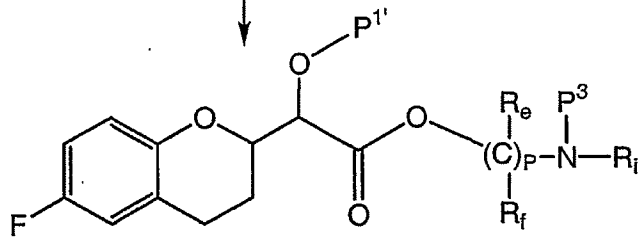

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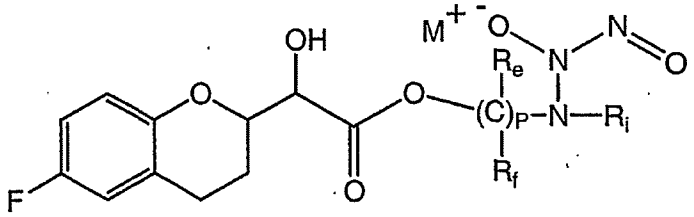

IIIH

Structure-Activity Relationship Studies of (±)-Terbutaline and (±)-Fenoterol on β_3-Adrenoceptors in the Guinea Pig Gastric Fundus

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Abstract

(±)-Terbutaline and (±)-fenoterol are both arylethanolamine analogs that have tert-butyl and aryliso-propyl substituents respectively at the α position on the nitrogen of the ethanolamine side chain. In the present study, we have investigated the structure-activity relationships of (±)-terbutaline and (±)-fenoterol as β_3-adrenoceptor agonists in the guinea pig gastric fundus. (±)-Terbutaline and (±)-fenoterol induced concentration-dependent relaxation of the precontracted gastric fundus with pD_2 values of 4.45 ± 0.10 and 5.90 ± 0.09, and intrinsic activities of 1.00 ± 0.03 and 0.99 ± 0.01 respectively. The combination of the selective β_1-adrenoceptor antagonist (±)-atenolol (100 μM), and the selective β_2-adrenoceptor antagonist (±)-butoxamine (100 μM), produced a 2 and 6 fold rightward shift of the concentration-response curves for (±)-terbutaline and (±)-fenoterol respectively, without depressing the maximal responses. The order of potency of these agonists was (pD_2 value): (±)-fenoterol (5.09 ± 0.10) > (±)-terbutaline (4.13 ± 0.08). In the presence of (±)-atenolol and (±)-butoxamine, however, the non-selective β_1, β_2- and β_3-adrenoceptor antagonist (±)-bupranolol caused a concentration-dependent rightward shift of the concentration-response curves for (±)-terbutaline and (±)-fenoterol. Schild plot analyses of the effects of (±)-bupranolol against these agonists gave pA_2 values of 6.21 ± 0.07 ((±)-terbutaline) and 6.37 ± 0.06 ((±)-fenoterol) respectively, and the slopes of the Schild plot were not significantly different from unity ($p>0.05$). These results suggest that the relaxant responses to (±)-terbutaline and (±)-fenoterol are mainly mediated through β_3-adrenoceptors in the guinea pig gastric fundus. The β_3-adrenoceptor agonist potencies of arylethanolamine analogs depend on the size of the end of the alkylamine side chain.

Key words: atypical β-adrenoceptor, β_3-adrenoceptor, structure-activity relationship, β_2-adrenoceptor agonist, Guinea pig gastric fundus

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Introduction

β-Adrenoceptors were initially classified into β_1- and β_2-adrenoceptor subtypes by Lands *et al.* (1967a; 1967b) based on the relative potencies of sympathomimetic amines and tissue localization. Functional and molecular cloning studies have indicated the presence of atypical β-adrenoceptors or β_3-adrenoceptors, that differ from the classical β_1- and β_2-adrenoceptors (for review see Arch and Kaumann, 1993). Atypical β-adrenoceptors, including β_3-adrenoceptors, mediated relaxation in gastrointestinal smooth muscle from a variety of species including guinea pig, rat, rabbit, and man (for review see Manara *et al.*, 1995b). The responses mediated through β_3-adrenoceptors are characterized by the following four criteria: (i) low sensitivity to classical β_1- and β_2-adrenoceptors antagonists (e.g., propranolol), (ii) stimulation by selective β_3-adrenoceptor agonists (e.g., BRL37344), (iii) stimulation by non-conventional partial β_3-adrenoceptor agonists (e.g., CGP12177A), and (iv) sensitivity to either selective β_3-adrenoceptor antagonists (e.g., SR59230A) (Arch and Kaumann, 1993; Manara *et al.*, 1995a; Kaumann and Molenaar, 1996) or the non-selective β_1-, β_2-, and β_3-adrenoceptor antagonists (e.g., bupranolol) (Kaumann, 1989).

In the guinea pig gastric fundus, Coleman *et al.* (1987) reported that relaxant responses to isoprenaline and noradrenaline were resistant to propranolol. Recently, we showed that β-adrenoceptors of the guinea pig gastric fundus fulfill all four criteria (Horinouchi and Koike, 1999; Horinouchi *et al.*, 2001d) and we described such β-adrenoceptors as 'β_3-adrenoceptors'. Furthermore, arylethanolamines (e.g., (–)-isoprenaline and BRL37344) and aryloxypropanolamines (e.g., (±)-CGP12177A, (±)-pindolol, (±)-carteolol and SR59230A) produced β_3-adrenoceptor mediating relaxation in the guinea pig gastric fundus (Horinouchi and Koike, 1999; 2000; 2001a; 2001b).

(±)-Terbutaline and (±)-fenoterol, selective β_2-adrenoceptor agonists, have tert-butyl and aryliso-propyl substituents respectively at the α position on the nitrogen of the ethanolamine side chain (see Fig. 1 for chemical structure). These drugs have an asymmetric carbon (Fig. 1). A mixture of equal parts of its stereoisomers is used for the treatment of bronchial asthma and in animal experiments. In addition, (±)-terbutaline and (±)-fenoterol bear a structural resemblance to (–)-isoprenaline and BRL37344 respectively. Therefore, we considered that (±)-terbutaline and (±)-fenoterol could also possess β_3-adrenoceptor agonistic activity in the guinea pig gastric fundus.

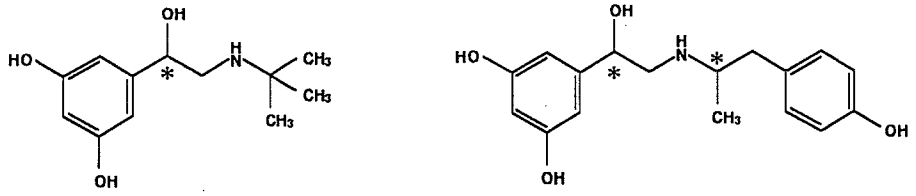

Fig. 1. Chemical structures of terbutaline and fenoterol used in the present study. The presence of the asymmetric carbon atom is denoted by *.

The aim of the present study was to clarify whether the relaxant responses to (±)-terbutaline and (±)-fenoterol are mediated by β_3-adrenoceptors in the guinea pig gastric fundus, and to establish the chemical requirements for β_3-adrenoceptor agonists by comparing the potencies of (±)-terbutaline and (±)-fenoterol.

Materials and Methods

Animals and tissue preparation

Male Hartley guinea pigs weighing 300–500 g (Saitama Experimental Animals Co., Ltd, Saitama, Japan) were used in accordance with the Guide for the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan), and the protocol of the present study was approved by the Institutional Animal Care and Use Committee. Guinea pigs were housed under standard laboratory conditions on a 12-h light/dark cycle (lights on 8:00 a.m.; lights off 8:00 p.m.) in a temperature (20–22°C) and relative humidity (50 ± 5%) controlled room. Food and water were available *ad libitum*.

Guinea pigs were sacrificed and the gastric fundus was isolated. The stomach contents were removed immediately and the connective tissue was dissected away. The gastric fundus was opened and cut into strips (4–6 mm wide, 15–20 mm long) running parallel to the longitudinal smooth muscle fibers and the gastric mucosae was carefully removed from the muscle layer. Strips were mounted vertically under an initial tension of 0.5 g in a 20-ml organ bath containing Ringer-Locke solution (NaCl, 154; KCl, 5.6; $CaCl_2$, 2.2; $MgCl_2$, 2.1; $NaHCO_3$, 5.9 and glucose, 2.8 mM), maintained at 32°C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Imipramine (1 μM, a neuronal uptake inhibitor), normetanephrine (10 μM, an extraneuronal uptake inhibitor), phentolamine (10 μM, an α-adrenoceptor antagonist) and L-ascorbic acid (10 μM, to prevent oxidation of catecholamine) were present in the medium throughout all experiments.

Experimental protocols

After the preparations were allowed to equilibrate for 30 min in the absence of β-adrenoceptor antagonist, the preparations were contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 3 μM), which induced a contraction equal to 70–80% of the maximal $PGF_{2\alpha}$-induced contraction. A sustained plateau phase was observed approximately 30 min after the addition of $PGF_{2\alpha}$, then test drugs were added cumulatively. The β-adrenoceptor-mediated relaxations caused by the test drugs were determined by measuring the inhibition of the $PGF_{2\alpha}$-induced contraction. Firstly, concentration-response curves for (–)-isoprenaline (up to 3 μM) were generated as controls (100%). $PGF_{2\alpha}$ (3 μM) was added to the bath 30 min after washing out the drug, then test drugs were added cumulatively until a maximal relaxant response was observed. The relaxation induced by these drugs was expressed as a percentage of the maximal relaxation produced by the reference drug, (–)-isoprenaline (3 μM), in the absence of β-adrenoceptor antagonist.

In order to assess the antagonistic effects of the combination of (±)-atenolol, (±)-butoxamine

and (±)-bupranolol, each antagonist was added to the bath 30 min before the addition of $PGF_{2\alpha}$. (±)-Atenolol (100 μM; an aryloxypropanolamine analog), (±)-butoxamine (100 μM; an arylethanolamine analog) and (±)-bupranolol (≤10 μM; an aryloxypropanolamine analog) themselves did not induce the inhibition of the $PGF_{2\alpha}$-induced contraction (data not shown).

Data analysis

The results are expressed as the mean ± S.E.M. for the number (n) of experiments performed. Agonistic potency was expressed as the pD_2 value (Van Rossum, 1963). The intrinsic activity of each drug was calculated as the ratio of the maximal relaxation induced by each agonist to the maximal relaxation induced by (–)-isoprenaline (3 μM), the full agonist, in the absence of a β-adrenoceptor antagonist. The competitive antagonistic potency of (±)-bupranolol was expressed as the pA_2 value. It was calculated according to the method of Tallarida *et al.* (1979), which was originally described by Arunlakshana and Schild (1959). Statistical significance between two data sets was tested by the Student's t test. A P value of less than 0.05 was considered to be statistically significant.

Drugs

The following drugs were used: (–)-isoprenaline hydrochloride, (±)-terbutaline hemisulfate, (±)-fenoterol hydrobromide, imipramine hydrochloride, normetanephrine hydrochloride, (±)-butoxamine hydrochloride, L-ascorbic acid (Sigma-Aldrich Co., St. Louis, Mo., USA); phentolamine mesylate (Novartis, Basel, Switzerland); (±)-atenolol (Research Biochemicals International, Natick, Mass., USA); (±)-bupranolol hydrochloride (Kaken Pharmaceutical Co., Ltd, Tokyo, Japan) and prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical Co., Ltd, Osaka, Japan). The other chemicals used were of analytical grade. All drugs were dissolved in distilled water.

Results

Relaxant effects of (±)-terbutaline and (±)-fenoterol

(±)-Terbutaline and (±)-fenoterol induced concentration-dependent relaxations of the gastric fundus precontracted with $PGF_{2\alpha}$ (Fig. 2). These agonists were full agonists in this preparation producing the same maximum response as (–)-isoprenaline. The pD_2 values of (±)-terbutaline and (±)-fenoterol were 4.45 ± 0.10 (n=11) and 5.90 ± 0.09 (n=15) respectively, while intrinsic activities were 1.00 ± 0.03 and 0.99 ± 0.01 respectively. Pretreatment with the selective β_1-adrenoceptor antagonist, (±)-atenolol (100 μM), and the selective β_2-adrenoceptor antagonist, (±)-butoxamine (100 μM), shifted the concentration-response curves for (±)-terbutaline and (±)-fenoterol to the right without reducing the maximum response (p>0.05) (Fig. 2). In the presence of (±)-atenolol plus (±)-butoxamine, the pD_2 values for (±)-terbutaline and (±)-fenoterol were 4.13 ± 0.08 (n=15) and 5.09 ± 0.10 (n=10) respectively, while the intrinsic activities were 0.93 ± 0.03 and 0.93 ± 0.02 respectively. Under conditions where β_1- and β_2-adrenoceptors were blocked, the potency (pD_2 value) of (±)-fenoterol is significantly (p<0.05) higher than that of (±)-terbutaline on the guinea pig gastric fundus.

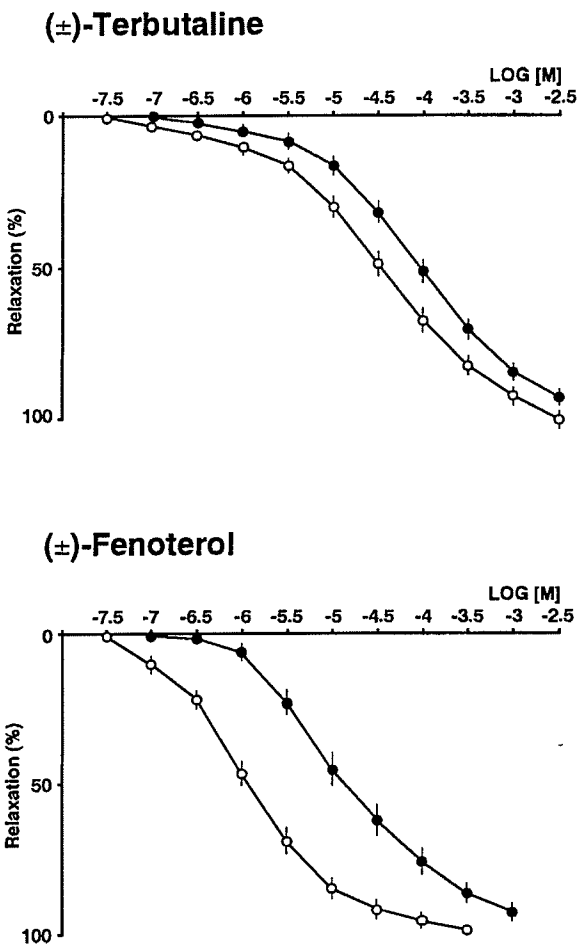

Fig. 2. The concentration-response curves to (±)-terbutaline and (±)-fenoterol in the absence (○) and presence (●) of (±)-atenolol (100 μM) plus (±)-butoxamine (100 μM) on the guinea pig gastric fundus. Ordinate: relaxation (%), expressed as a percentage of the maximum relaxation (in the absence of β-adrenoceptor antagonists) induced by (−)-isoprenaline (3 μM), abscissa: concentration (M) of the test drugs. Each point represents the mean ± S.E.M. of 10–15 experiments.

Effect of (±)-bupranolol on the relaxation to (±)-terbutaline and (±)-fenoterol

In the presence of (±)-atenolol (100 μM) and (±)-butoxamine (100 μM) to block β_1- and β_2-adrenoceptors, the non-selective β_1-, β_2- and β_3-adrenoceptor antagonist (±)-bupranolol (3 and 10 μM) produced concentration-dependent rightward shifts of concentration-response curves for (±)-terbutaline and (±)-fenoterol (Fig. 3). The Schild plot of the data revealed the pA_2 values for (±)-bupranolol against (±)-terbutaline and (±)-fenoterol to be 6.21 ± 0.07 and 6.37 ± 0.06 respectively. The slope of each regression line was not significantly different from unity (p>0.05) (Fig. 3). Since higher concentrations of (±)-terbutaline and (±)-fenoterol were needed, we could not use (±)-bupranolol (30 μM) in this study.

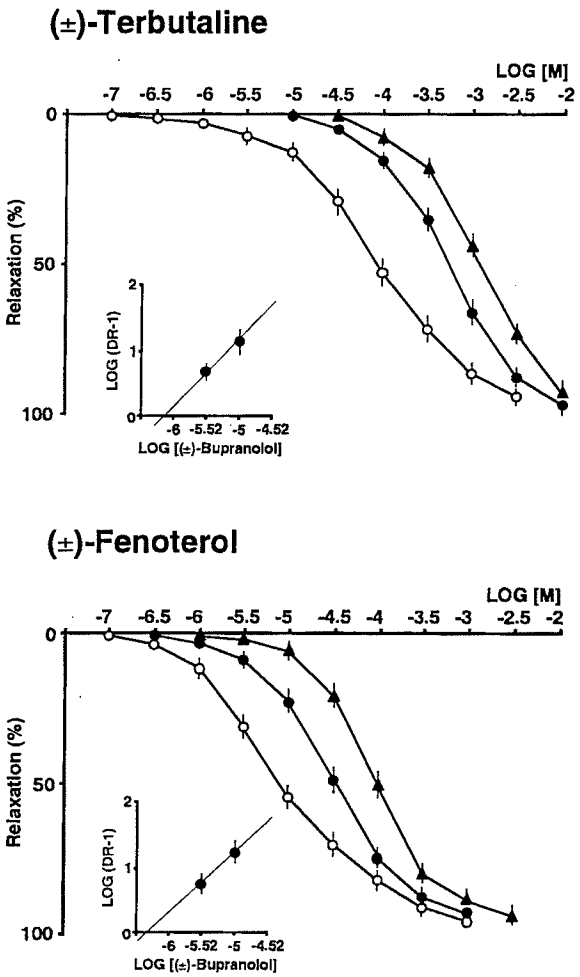

Fig. 3. Effects of (±)-bupranolol on concentration-response curves for (±)-terbutaline and (±)-fenoterol in the presence of (±)-atenolol (100 μM) plus (±)-butoxamine (100 μM) on the guinea pig gastric fundus. Control (○); (±)-bupranolol 3 μM (●); (±)-bupranolol 10 μM (▲). Ordinate: relaxation (%), expressed as a percentage of the maximum relaxation (in the absence of β-adrenoceptor antagonists) induced by (–)-isoprenaline (3 μM), abscissa: concentration (M) of the test drugs. Each point represents the mean ± S.E.M. of 6–8 experiments. The insets show the corresponding Schild plots.

Discussion

In the present study, the structure-activity relationships of (±)-terbutaline and (±)-fenoterol were examined on β_3-adrenoceptor agonistic activity in the guinea pig gastric fundus. It is generally accepted that (±)-terbutaline and (±)-fenoterol are potent and selective β_2-adrenoceptor agonists. The pD_2 values for (±)-terbutaline and (±)-fenoterol reported in the literature are 6.63 and 7.74 respectively for the guinea pig trachea contracted by 1 μM carbachol (Kuällström *et al.*, 1994). In the precontracted gastric fundus, (±)-terbutaline and (±)-fenoterol

induced concentration-dependent relaxations with pD_2 values of 4.45 ± 0.10 and 5.90 ± 0.09 respectively. Furthermore, relaxant responses to (±)-terbutaline and (±)-fenoterol were resistant to blockade by a combination of (±)-atenolol (100 μM) and (±)-butoxamine (100 μM) which normally blocks responses in preparation known to contain β_1- and β_2-adrenoceptors. Therefore, these results suggest that the relaxations in response to (±)-terbutaline and (±)-fenoterol are mainly mediated via β_3-adrenoceptors in the guinea pig gastric fundus.

To confirm the interaction of these agonists with β_3-adrenoceptors, we used the non-selective β_1-, β_2- and β_3-adrenoceptor antagonist (±)-bupranolol under conditions designed to assess only β_3-adrenoceptors (Horinouchi and Koike, 1999). In the presence of both (±)-atenolol and (±)-butoxamine, relaxant responses to (±)-terbutaline and (±)-fenoterol were antagonized by (±)-bupranolol. (±)-Bupranolol, at a concentration much higher than that which would antagonize β_1- and β_2-adrenoceptor-mediated effects, antagonized atypical β/β_3-adrenoceptors (Koike *et al.*, 1995; Kaumann and Molenaar, 1996; Malinowska and Schlicker, 1997; Horinouchi and Koike, 1999). These results suggest that (±)-terbutaline- and (±)-fenoterol-induced relaxations were solely mediated by β_3-adrenoceptors in the guinea pig gastric fundus when classical β-adrenoceptors are blocked.

The pA_2 values for (±)-bupranolol obtained in this study resemble those (approximately 6) against catecholamines reported in a previous study on the guinea pig gastric fundus (Horinouchi and Koike, 1999), whereas these values are larger than the pA_2 value (5.29) against (±)-carteolol (Horinouchi and Koike, 2000). (±)-Terbutaline, (±)-fenoterol and catecholamines are arylethanolamines, while (±)-carteolol is an aryloxypropanolamine. It is possible that the β_3-adrenoceptor antagonistic activities of (±)-bupranolol against arylethanolamine are more potent than those against aryloxypropanolamines.

(±)-Terbutaline and (±)-fenoterol have a tert-butyl and aryliso-propyl substituent respectively at the α position on the nitrogen of the ethanolamine side chain. Thus (±)-fenoterol has a structure close to that of (±)-terbutaline, except that the tert-butyl substituent is substituted by the aryliso-propyl substituent, which is a bulky group and may increase the steric bulk and lipophilicity at the end of the alkylamine chain. (±)-Fenoterol was 10-fold more potent than (±)-terbutaline, indicating that an increase in the size of the end of the side chain increases β_3-adrenoceptor agonistic activity in the guinea pig gastric fundus. These results support our previous report concerning atypical β-adrenoceptors of the guinea pig duodenum (Horinouchi and Koike, 2001c).

In summary, the relaxant responses to (±)-terbutaline and (±)-fenoterol, which are resistant to classical β-adrenoceptor antagonists, are predominantly mediated by β_3-adrenoceptors. The structure-activity relationship study of (±)-terbutaline and (±)-fenoterol indicates that arylethanolamines, which have a bulky group on the end of the alkylamine chain, exhibit high potency for β_3-adrenoceptors.

Acknowledgement

We are grateful to Kaken Pharmaceutical Co., Ltd (Tokyo, Japan) for the gift of (±)-bupranolol hydrochloride.

References

Arch, J.R.S. and Kaumann, A.J. (1993). β_3- and Atypical β-adrenoceptors. *Med. Res. Rev.* **13**: 663–729.

Arunlakshana, O. and Schild, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.* **14**: 48–58.

Coleman, R.A., Denyer, L.H. and Sheldrick, K.E. (1987). β-Adrenoceptors in guinea-pig gastric fundus - are they the same as the 'atypical' β-adrenoceptors in rat adipocytes? *Br. J. Pharmacol.* **90**: 40P.

Horinouchi, T. and Koike, K. (1999). Pharmacological analysis of atypical β-adrenoceptors in the guinea pig gastric fundus using the β_3-adrenoceptor antagonist bupranolol. *Pharmacology* **59**: 290–297.

Horinouchi, T. and Koike, K. (2000). Partial agonistic effects of carteolol on atypical β-adrenoceptors in the guinea pig gastric fundus. *Jpn. J. Pharmacol.* **84**: 287–292.

Horinouchi, T. and Koike, K. (2001a). Agonistic activity of SR59230A at atypical β-adrenoceptors in guinea pig gastric fundus and duodenum. *Eur. J. Pharmacol.* **416**: 165–168.

Horinouchi, T. and Koike, K. (2001b). (±)-Pindolol acts as a partial agonist at atypical β-adrenoceptors in the guinea pig duodenum. *Jpn. J. Pharmacol.* **85**: 35–40.

Horinouchi, T. and Koike, K. (2001c). Functional properties of atypical β-adrenoceptors on the guinea pig duodenum. *Eur. J. Pharmacol.* **416**: 153–163.

Horinouchi, T., Yamamoto, Y. and Koike, K. (2001d). Effect of SR59230A on atypical β-adrenoceptor mediating relaxation in the guinea pig gastric fundus. *Pharmacology* **262**: 98–102.

Kaumann, A.J. (1989). Is there a third heart β-adrenoceptor? *Trends Pharmacol. Sci.* **10**: 316–320.

Kaumann, A.J. and Molenaar, P. (1996). Differences between the third cardiac β-adrenoceptor and the colonic β_3-adrenoceptor in the rat. *Br. J. Pharmacol.* **118**: 2085–2098.

Koike, K., Horinouchi, T. and Takayanagi, I. (1995). Possible mechanisms of β-adrenoceptor-mediated relaxation induced by noradrenaline in guinea pig taenia caecum. *Eur. J. Pharmacol.* **279**: 159–163.

Kuällström, B.L., Sjöberg, J. and Waldeck, B. (1994). The interaction between salmeterol and β_2-adrenoceptor agonists with higher efficacy on guinea-pig trachea and human bronchus in vitro. *Br. J. Pharmacol.* **113**: 687–692.

Lands, A.M., Arnold, A., McAuliff, J.P., Luduena, F.P. and Brown Jun, T.G. (1967a). Differentiation of receptor systems activated by sympathomimetic amines. *Nature* **214**: 597–598.

Lands, A.M., Luduena, F.P. and Buzzo, H.J. (1967b). Differentiation of receptors responsive to isoproterenol. *Life Sci.* **6**: 2241–2249.

Malinowska, B. and Schlicker, E. (1997). Further evidence for differences between cardiac atypical β-adrenoceptors and brown adipose tissue β_3-adrenoceptors in pithed rat. *Br. J. Pharmacol.* **122**: 1307–1314.

Manara, L., Badone, D., Baroni, M., Boccardi, G., Cecchi, R., Croci, T., Giudice, A., Guzzi, U. and Le Fur, G. (1995a). Aryloxypropanolaminotetralines are the first selective antagonists for atypical (β_3) β-adrenoceptors. *Pharmacol. Commun.* **6**: 253–258.

Manara, L., Croci, T. and Landi, M. (1995b). β_3-Adrenoceptors and intestinal motility. *Fund. Clin. Pharmacol.* **9**: 332–342.

Tallarida, R.J., Cowan, A. and Adler, M.W. (1979). pA_2 and receptor differentiation: A statistical analysis of competitive antagonism. *Life Sci.* **25**: 637–654.

Van Rossum, J.M. (1963). Cumulative dose-response curves, II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn.* **143**: 299–330.

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Involvement of the β_3 Adrenoceptor in Nebivolol-Induced Vasorelaxation in the Rat Aorta

Annemieke A. de Groot, Marie-Jeanne Mathy, Pieter A. van Zwieten, and Stephan L. M. Peters

Abstract: Nebivolol is a highly selective β_1 adrenoceptor blocker with additional vasodilating properties. Although it has been shown that the nebivolol-induced vasorelaxation is nitric oxide (NO) and cGMP dependent, the receptor that mediates these actions remains controversial, and serotonergic as well as β-adrenergic pathways may be involved. Therefore, functional experiments investigating the receptor involved in nebivolol-induced vasorelaxation were performed in the rat aorta. Isolated aortic rings were exposed to cumulative concentrations of nebivolol. Nebivolol concentrations of 3 μmol/L and higher caused vasorelaxation, which was inhibited by the presence of the NO synthase inhibitor L-NNA (100 μmol/L), or by mechanical removal of the endothelium. Exposure of the vessel rings to the selective 5-HT_{1A} antagonist NAN-190 (1 μmol/L) or the 5-$HT_{1/2}$ antagonist methysergide (1 μmol/L) did not influence nebivolol-induced vasorelaxation. Similarly, the incubation with the β_2-adrenoceptor antagonist butoxamine (50 μmol/L) did not prevent vasorelaxation. The selective β_3-adrenoceptor antagonist S-(−)-cyanopindolol (1 μmol/L), however, significantly counteracted the nebivolol-induced vasorelaxation. Furthermore, exposure of the aortic rings to cumulative concentrations of the β_3 selective adrenoceptor agonist BRL37344 caused, like nebivolol, NO-dependent vasorelaxation that was antagonized by S-(−)-cyanopindolol. The results suggest that nebivolol-induced NO-dependent vasorelaxation is, at least in part, caused by a β_3-adrenoceptor agonistic effect.

Key Words: nebivolol, rat aorta, vasorelaxation, nitric oxide, β-adrenergic receptor, serotonergic receptor

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Nebivolol, a racemic mixture of D- and L- enantiomers, is known to be the most β_1-selective adrenoceptor antagonist available today.[1,2] Interestingly, its hemodynamic profile differs from that of classic β-adrenoceptor antagonists as a result of an additional vasodilating action, which has been demonstrated both in animals[3] and in humans.[4–6]

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The mechanism of the vasodilator action of nebivolol is currently under investigation. Its vasodilating properties are mainly caused by its L-enantiomer, while the β_1 blocking effects are supposedly associated with its D-enantiomer.[7] Nebivolol-induced vasodilation is endothelium dependent and can be inhibited by blockers of nitric oxide synthase (NOS) and soluble guanylate cyclase, thus suggesting that endothelial release of nitric oxide (NO) mediates this process.[3–5,8]

However, nebivolol appears to provoke differential effects in various vascular beds. Altwegg et al[9] described that nebivolol induces NO-mediated relaxations in small mesenteric but not in large elastic vessels in the rat. Broeders et al,[10] however, showed that the mouse aorta did produce NO after exposure to nebivolol, but only after its biodegradation in vivo. Certain functional studies indicate that nebivolol causes NO-dependent vasorelaxation in rat aortic rings, without primary metabolization in vivo.[8] Furthermore, the receptor by which the vasodilating action of nebivolol is mediated and the exact second messenger pathway involved remain to be defined. Nebivolol is devoid of α-adrenergic antagonist activity. However, several other receptor-mediated pathways, such as the serotonergic pathway, have been proposed to play a role. Although Kakoki et al[8] observed that in the rat aorta NAN-190, a 5-HT_{1A} antagonist, was able to block the vasodilatory effects of nebivolol, in the canine coronary artery its vasodilating action was not inhibited by the nonselective 5-HT_1 and 5-HT_2 blocker methysergide.[3]

The involvement of β_2- as well as β_3-adrenoceptor subtypes has been suggested. Broeders et al[10] concluded, after the aforementioned in vitro and in vivo experiments in the mouse aorta, that in vivo metabolized nebivolol increased NO production via the activation of the β_2 receptor, followed by a subsequent rise in intracellular calcium and NOS-dependent NO production. In contrast to these findings, in studies with human umbilical vein endothelial cells performed by Gosgnach et al,[11] the β_2 receptor appears not to be involved in nebivolol-induced NO production. Their findings suggest that nebivolol could behave as a β_3-receptor agonist and consequently cause NO-dependent vasorelaxation. As these experiments were performed under in vitro conditions, the effects observed were caused by nebivolol, but not by one of its metabolites. So far, the influence of the β_3 adrenergic pathway on

nebivolol-induced vasorelaxation has not yet been studied in a functional model. Accordingly, the involvement of β-adrenergic as well as serotonergic mechanisms have been described in various different models and vascular beds, but the pathway by which nebivolol induces vasodilatation remains to be established in detail.

For this reason, we aimed to investigate the role of several receptors that have been suggested to be involved, such as β-adrenoceptors (β_2 and β_3) as well as serotonergic receptors (5-HT_1 and 5-HT_2). All receptor-mediated pathways were explored in the same functional model, using rat aortic rings in an organ-bath setup.

MATERIALS AND METHODS

Thoracic Aorta Preparation and Priming Procedure

Male Wistar Kyoto rats (Charles River, Germany) weighing 240 to 260 g were anesthetized using a combination of ketamine (40 mg i.p.) and xylazine (4 mg i.p.). The thoracic aorta was carefully dissected and placed immediately in oxygenated Krebs-Henseleit solution (composition in mmol/L: 118.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.1 KH_2PO_4, and 5.6 glucose) at room temperature. The vessel was cleaned of adhesive fat and connective tissue, care being taken not to stretch the vessel and not to damage the endothelium or smooth muscle cells. The isolated vessel was cut into rings approximately 3 mm in length.

For the recording of tension the vessel rings were mounted, between 2 L-shaped stainless steel hooks, in 5-mL organ baths filled with oxygenated Krebs-Henseleit solution at 37.8°C (pH 7.4). Each preparation was fixed, via a silk thread, to an isometric force transducer (A.D. Instruments, Castle Hill, Australia) and force was recorded via a MacLab/8 computer system (A.D. Instruments). Each vessel ring was subjected to an initial resting tension of 9.8 mN that was maintained throughout.

After an equilibration period of 45 minutes, the vascular rings were primed and tested for viability by exposing them three times to a depolarizing 40-mmol/L potassium chloride (KCl) solution. The KCl solution had the same composition as the Krebs-Henseleit solution used, except for sodium chloride, which had been partially replaced by an equimolar amount of KCl corresponding to a total concentration of 40-mmol/L KCl. After each depolarization the medium was exchanged and the vessel rings were allowed to equilibrate for 20 minutes. Between the second and the third KCL-induced vasoconstriction, the endothelial function of the vessel rings was tested by applying a precontraction with phenylephrine (1 μmol/L) followed by a single concentration (1 μmol/L) of metacholine. Vessel rings with an endothelial function ≥80% were regarded as vessels with an intact endothelium and included in the experiments. When endothelium was deliberately removed, only vessels with an endothelial function of ≤20% were regarded as endothelium-denuded and included in further experiments.

Experimental Protocol

Following the priming procedure, the vessels were subjected to either the NOS inhibitor L-NNA (100 μmol/L), the 5-HT_{1A} antagonist NAN-190 (1 μmol/L), the 5-$HT_{1A/1B}$ receptor antagonist methysergide (1 μmol/L), or to the β_2 and β_3 adrenoceptor antagonists butoxamine (50 μmol/L) and S-(−)-cyanopindolol (1 μmol/L), respectively. Other vessels were not incubated and served as controls. Thirty minutes of incubation was followed by precontraction of the vessels with the thromboxane A_2 analogue U46 (30 nmol/L) and after the contractile response had reached a plateau, cumulative concentrations of dl-nebivolol were added (100 nmol/L to 10 μmol/L). Other vessel rings were subjected to cumulative concentrations of metoprolol, a selective β_1 adrenoceptor antagonist devoid of vasodilating properties (100 nmol/L to 10 μmol/L). Furthermore, cumulative concentrations of the selective β_3-adrenoceptor agonist BRL 37344 (1 nmol/L to 10 μmol/L) were applied, in the presence and absence of L-NNA. In one series of experiments, the endothelium was mechanically removed, by gently rubbing the intima of the vessels three times gently with a cannula.

Statistical Evaluation

All responses were calculated as percentage of the vascular tone reached by preconstriction with 30-nmol/L U46. Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA), followed by the Dunnett post test for comparison versus control, or the *t* test were performed. *P* values less than 0.05 were considered to be statistically significant.

Chemicals

L-NNA (N^{ω}-nitro-L-arginine) was obtained from Alexis Biochemicals (San Diego, CA, U.S.A.). NAN-190 (1-(2-methoxyphenyl)-4-(4-phthalimidobutyl) piperazine) hydrobromide, methysergide ([8b(S)]-9,10-didehydro-N-[1-(hydroxymethyl)propyl]-1,6-dimethylergoline-8-carboxamide) maleate and BRL37344 [(*R**,*R**)-(±)-4-[2-[(2-(3-chlorophenyl)- 2-hydroxyethyl)amino]propyl] phenoxyacetic acid] sodium salt were obtained from Tocris (Ellisville, MO, U.S.A.). Phenylephrine [(R)-(−)-1-(3-hydroxyphenyl)-2-methylaminoethanol] hydrochloride, metacholine (acetyl-β-methylcholine) chloride, U46619 (9,11-dideoxy-11a, 9a-epoxymethanoprostaglandin F_{2a}), metoprolol (1-(isopropylamino)-3-(p-[β-methoxyethyl]phenoxy)-2-propanol) tartrate, butoxamine (α-(1-[t-butylamino]ethyl)-2,5-dimethoxybenzyl alcohol) hydrochloride and S-(−)-cyanopindolol (4-(3-[(1,1-dimethylethyl)amino]-2-hydroxypropyl)-1H-indole-2-carbonitrile) hemifumarate were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

dl-Nebivolol (α, α'-[iminobis(methylene)]-bis-[6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol]) was a kind gift from the Menarini Group (Florence, Italy). All compounds were dissolved in distilled water, except for nebivolol, which was dissolved in DMSO and further diluted. The organ bath concentration of DMSO never exceeded 0.002%. In this concentration, DMSO showed no pharmacological effect (data not shown).

RESULTS

In the isolated rat thoracic aorta, nebivolol, in concentrations of 3 μmol/L and higher, produced a concentration-dependent vasodilatation. In the highest concentration applied (10 μmol/L), this resulted in a vascular tone of 55.2% ± 7.8% of the applied precontraction. In similar concentrations, metoprolol did not cause vasodilatation (Fig. 1).

As shown in Figure 2, the vasodilator effect of nebivolol, in concentrations of 3 and 10 μmol/L, was significantly blocked by exposure to 100 μmol/L of the NOS inhibitor L-NNA (102.3% ± 2.3% versus 84.6% ± 4.2% and 99%.1 ± 2.2% versus 55.2% ± 7.8%, respectively; $P < 0.05$, n = 6–13). Mechanical removal of endothelium had the same effect (98.0% ± 0.9% versus 84.6% ± 4.2% and 84.62% ± 1.8% versus 55.2% ± 7.8%, respectively; $P < 0.05$, n = 6–13).

To investigate whether serotonergic pathways play a role in the observed nebivolol-induced vasorelaxation, the vessel rings were exposed to the 5-HT_{1A} specific receptor antagonist NAN-190 and to the 5-$HT_{1/2}$ receptor antagonist methysergide. Neither NAN-190 (1 μmol/L) nor methysergide (1 μmol/L) significantly affected nebivolol-induced vasorelaxation (Fig. 3).

In addition, the involvement of β-adrenergic pathways was investigated. Specific β_2-adrenoceptor blockade with butoxamine (50 μmol/L) did not influence the vasodilatory effect of nebivolol. However, the addition of 1-μmol/L S-(−)-cyanopindolol significantly affected the response to 10-μmol/L nebivolol and prevented nebivolol-induced vasorelaxation (82.0% ± 4.5% versus 55.2% ± 7.8%; $P < 0.05$, n = 6–13) (Fig. 4).

Figure 5 shows responses to the specific β_3 agonist BRL 37344, applied to confirm that β_3 adrenoceptor stimulation leads to NO-dependent vasorelaxation in the rat thoracic aorta. Indeed, BRL 37344 produced vasodilatation that was significantly blocked by L-NNA (Fig. 5). In a concentration of 1-μmol/L BRL 37344, L-NNA (100 μmol/L) increased vascular tone from 81.6% ± 5.5% to 98.8% ± 0.5% ($P < 0.05$; n = 6) and in the maximal concentration of BRL 37344 applied (10 μmol/L), from 45.1% ± 9.0% to 83.9% ± 6.2% ($P < 0.05$, n = 6). β_3 adrenoceptor blockade completely inhibited BRL 37344-induced vasorelaxation: 97.5% ± 0.8% (S-(−)-cyanopindolol) versus 81.6 ± 5.5% (control) in a concentration of 1-μmol/L BRL 37344 and 94.7% ± 1.7% (S-(−)-cyanopindolol) versus 45.1% ± 9.0% (control) in a concentration of 10-μmol/L BRL 37344 ($P < 0.05$, n = 6).

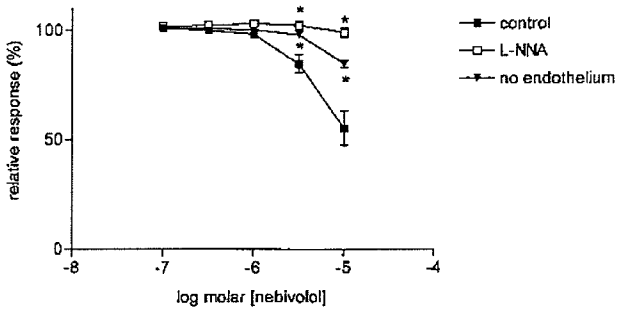

FIGURE 2. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol under control conditions (■), after incubation with 100-μmol/L L-NNA (□) and after mechanical removal of the endothelium (▼). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus control.

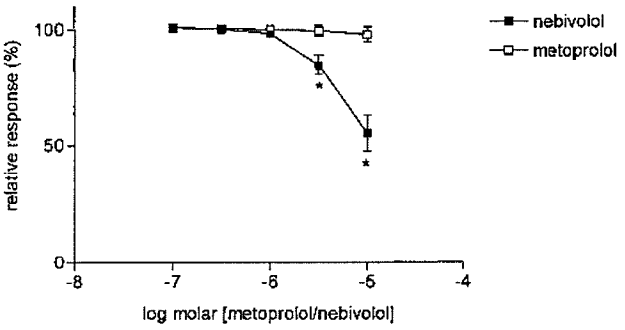

FIGURE 1. Vasodilator activity of nebivolol. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol (■) and metoprolol (□). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus metoprolol.

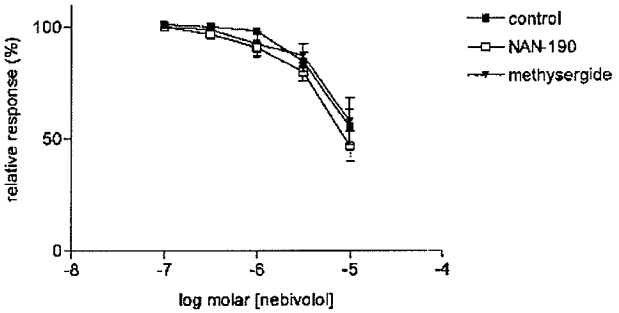

FIGURE 3. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol under control conditions (■), after incubation with 1-μmol/L NAN-190 (□), and after incubation with 1-μmol/L methysergide (▼). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus control.

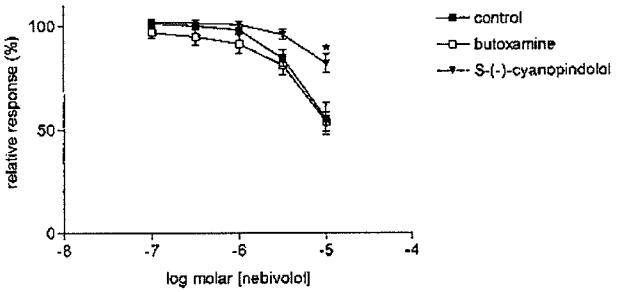

FIGURE 4. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol under control conditions (■), after incubation with 50-μmol/L butoxamine (□), and after incubation with 1-μmol/L S-(−)-cyanopindolol (▼). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus control.

DISCUSSION

In the present study, we investigated the vasodilating properties of nebivolol in the rat aorta. Nebivolol produced endothelium/NO-dependent vasodilatation in similar concentrations as applied in previous studies.[3,8] In the same concentration range, metoprolol, a β_1-adrenoceptor antagonist without NO-releasing activity, did not cause vasorelaxation.

In an organ bath model similar to ours, Altwegg et al[9] did not observe nebivolol-induced vasorelaxation in the aorta. However, our results are in accordance with those of Kakoki et al,[8] who also reported endothelium-dependent vasodilating effects of nebivolol in rat aortic rings. In contrast to our results, in their study, the 5-HT_1 blocker NAN-190 (100 nmol/L) was able to inhibit vasorelaxation by nebivolol. It is worth mentioning that, in experiments performed in our model, NAN-190, in a concentration as low as 1 nmol/L, attenuated phenylephrine-induced vasoconstriction and in higher concentrations (10 and 100 nmol/L) even abolished the α_1-adrenoceptor–mediated contractile response completely (data not shown). These findings imply that NAN-190 cannot be regarded as specific for 5-HT_1 receptors: it blocks α_1-adrenoceptors and possibly activates or deactivates other adrenergic receptors. The response to U46, the thromboxane A_2-analogue we applied as a preconstrictor agent, was not affected by incubation with NAN-190. In these experiments, NAN-190 did not influence nebivolol-induced vasodilatation. However, the specificity of NAN-190 remains questionable and therefore results obtained with the 5-HT_1 and 5-HT_2 blocker methysergide, that in the same concentration range did not affect phenylephrine-induced vasoconstriction, appear to be more conclusive. Previous studies performed with methysergide in the canine coronary artery showed no serotonergic involvement in nebivolol-induced vasorelaxation.[3] We demonstrated that methysergide does not affect nebivolol-induced vasorelaxation in the rat aorta, suggesting that the NO-dependent dilating properties of nebivolol are not mediated by a serotonergic pathway.

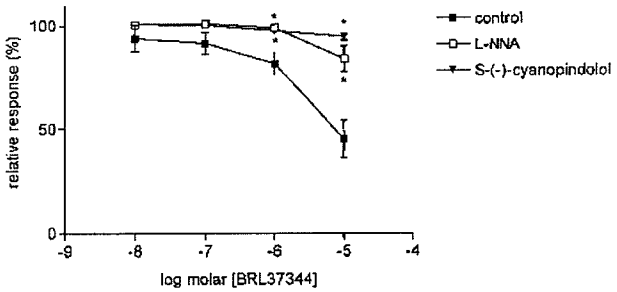

FIGURE 5. Vasodilator activity of BRL 37344. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of BRL 37344 under control conditions (■), after incubation with 100-μmol/L L-NNA (□), and after incubation with 1 μM S-(−)-cyanopindolol (▼). Data are expressed as mean ± SEM (n = 6). *$P < 0.05$ versus control.

We therefore hypothesized that nebivolol might exert its NO-mediated vasodilating action through a β_2- or β_3-adrenoceptor–mediated pathway. To investigate both pathways, the vessel rings were subjected to the β_2-adrenoceptor antagonist butoxamine and the β_3-adrenoceptor antagonist S-(−)-cyanopindolol. S-(−)-cyanopindolol significantly attenuated the vasorelaxation by nebivolol. β_2 blockade with butoxamine did not affect the concentration response curve. These results suggest that in the rat aorta, NO-dependent vasorelaxation by nebivolol may be mediated by β_3 receptor stimulation. This presumption is in accordance with the findings of Gosgnach et al,[11] who analyzed the different signaling pathways implicated in the response of human umbilical vein endothelial cells to nebivolol. They suggest that nebivolol possesses β_3-adrenoceptor agonistic properties that seem to be responsible for its endothelium-dependent vasodilating activity.

Most studies on the β_3-adrenoceptor subtype have focused on its control of lipolysis in adipose tissues. However, more recent studies have investigated the involvement of β_3-adrenoceptors in the physiological control of cardiac and vascular contractility. These studies have produced pharmacological and molecular evidence that supports the functional role of β_3-adrenoceptors in cardiovascular tissues of various species.[12] Furthermore, they have demonstrated that β_3 receptor activation indeed causes relaxation both in cardiac and in vascular tissue via NOS activation leading to release of NO.[13]

To confirm that β_3 adrenoceptor activation causes endothelium-dependent vasorelaxation in our model, we exposed aortic rings to cumulative concentrations of BRL 37344, a specific β_3 adrenoceptor agonist. Indeed, BRL 37344 produced concentration-dependent relaxations that were inhibited by L-NNA as well as by β_3-adrenoceptor blockade with S-(−)-cyanopindolol.

CONCLUSION

Based on the data presented in this functional study, we conclude that, in the rat aorta, nebivolol-induced NO-

dependent vasorelaxation is, at least in part, caused by a β_3-agonistic effect.

REFERENCES

1. Pessina AC. Metabolic effects and safety profile of nebivolol. *J Cardiovasc Pharmacol.* 2001;38(suppl 3):S33–S35.
2. Ritter JM. Nebivolol: endothelium-mediated vasodilating effect. *J Cardiovasc Pharmacol.* 2001;38(suppl 3):S13–S16.
3. Gao YS, Nagao T, Bond RA, et al. Nebivolol induces endothelium-dependent relaxations of canine coronary arteries. *J Cardiovasc Pharmacol.* 1991;17:964–969.
4. Bowman AJ, Chen CP, Ford GA. Nitric oxide mediated venodilator effects of nebivolol. *Br J Clin Pharmacol.* 1994;38:199–204.
5. Cockcroft JR, Chowienczyk PJ, Brett SE, et al. Nebivolol vasodilates human forearm vasculature: evidence for an L-arginine/NO-dependent mechanism. *J Pharmacol Exp Ther.* 1995;274:1067–1071.
6. Dawes M, Brett SE, Chowienczyk PJ, et al. The vasodilator action of nebivolol in forearm vasculature of subjects with essential hypertension. *Br J Clin Pharmacol.* 1999;48:460–463.
7. Van Nueten L, De Cree J. Nebivolol: comparison of the effects of dl-nebivolol, d-nebivolol, l-nebivolol, atenolol, and placebo on exercise-induced increases in heart rate and systolic blood pressure. *Cardiovasc Drugs Ther.* 1998;12:339–344.
8. Kakoki M, Hirata Y, Hayakawa H, et al. Effects of vasodilatory beta-adrenoceptor antagonists on endothelium- derived nitric oxide release in rat kidney. *Hypertension.* 1999;33:467–471.
9. Altwegg LA, d'Uscio LV, Barandier C, et al. Nebivolol induces NO-mediated relaxations of rat small mesenteric but not of large elastic arteries. *J Cardiovasc Pharmacol.* 2000;36:316–320.
10. Broeders MA, Doevendans PA, Bekkers BC, et al. Nebivolol: a third-generation beta-blocker that augments vascular nitric oxide release: endothelial beta(2)-adrenergic receptor-mediated nitric oxide production. *Circulation.* 2000;102:677–684.
11. Gosgnach W, Boixel C, Nevo N, et al. Nebivolol induces calcium-independent signaling in endothelial cells by a possible beta-adrenergic pathway. *J Cardiovasc Pharmacol.* 2001;38:191–199.
12. Gauthier C, Langin D, Balligand JL. Beta3-adrenoceptors in the cardiovascular system. *Trends Pharmacol Sci.* 2000;21:426–431.
13. Trochu JN, Leblais V, Rautureau Y, et al. Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta. *Br J Pharmacol.* 1999;128:69–76.

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Key Words: nebivolol, rat aorta, vasorelaxation, nitric oxide, β-adrenergic receptor, serotonergic receptor

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Nebivolol, a racemic mixture of D- and L- enantiomers, is known to be the most β_1-selective adrenoceptor antagonist available today.[1,2] Interestingly, its hemodynamic profile differs from that of classic β-adrenoceptor antagonists as a result of an additional vasodilating action, which has been demonstrated both in animals[3] and in humans.[4–6]

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The mechanism of the vasodilator action of nebivolol is currently under investigation. Its vasodilating properties are mainly caused by its L-enantiomer, while the β_1 blocking effects are supposedly associated with its D-enantiomer.[7] Nebivolol-induced vasodilation is endothelium dependent and can be inhibited by blockers of nitric oxide synthase (NOS) and soluble guanylate cyclase, thus suggesting that endothelial release of nitric oxide (NO) mediates this process.[3–5,8]

However, nebivolol appears to provoke differential effects in various vascular beds. Altwegg et al[9] described that nebivolol induces NO-mediated relaxations in small mesenteric but not in large elastic vessels in the rat. Broeders et al,[10] however, showed that the mouse aorta did produce NO after exposure to nebivolol, but only after its biodegradation in vivo. Certain functional studies indicate that nebivolol causes NO-dependent vasorelaxation in rat aortic rings, without primary metabolization in vivo.[8] Furthermore, the receptor by which the vasodilating action of nebivolol is mediated and the exact second messenger pathway involved remain to be defined. Nebivolol is devoid of α-adrenergic antagonist activity. However, several other receptor-mediated pathways, such as the serotonergic pathway, have been proposed to play a role. Although Kakoki et al[8] observed that in the rat aorta NAN-190, a 5-HT_{1A} antagonist, was able to block the vasodilatory effects of nebivolol, in the canine coronary artery its vasodilating action was not inhibited by the nonselective 5-HT_1 and 5-HT_2 blocker methysergide.[3]

The involvement of β_2- as well as β_3-adrenoceptor subtypes has been suggested. Broeders et al[10] concluded, after the aforementioned in vitro and in vivo experiments in the mouse aorta, that in vivo metabolized nebivolol increased NO production via the activation of the β_2 receptor, followed by a subsequent rise in intracellular calcium and NOS-dependent NO production. In contrast to these findings, in studies with human umbilical vein endothelial cells performed by Gosgnach et al,[11] the β_2 receptor appears not to be involved in nebivolol-induced NO production. Their findings suggest that nebivolol could behave as a β_3-receptor agonist and consequently cause NO-dependent vasorelaxation. As these experiments were performed under in vitro conditions, the effects observed were caused by nebivolol, but not by one of its metabolites. So far, the influence of the β_3 adrenergic pathway on

nebivolol-induced vasorelaxation has not yet been studied in a functional model. Accordingly, the involvement of β-adrenergic as well as serotonergic mechanisms have been described in various different models and vascular beds, but the pathway by which nebivolol induces vasodilatation remains to be established in detail.

For this reason, we aimed to investigate the role of several receptors that have been suggested to be involved, such as β-adrenoceptors (β_2 and β_3) as well as serotonergic receptors (5-HT_1 and 5-HT_2). All receptor-mediated pathways were explored in the same functional model, using rat aortic rings in an organ-bath setup.

MATERIALS AND METHODS

Thoracic Aorta Preparation and Priming Procedure

Male Wistar Kyoto rats (Charles River, Germany) weighing 240 to 260 g were anesthetized using a combination of ketamine (40 mg i.p.) and xylazine (4 mg i.p.). The thoracic aorta was carefully dissected and placed immediately in oxygenated Krebs-Henseleit solution (composition in mmol/L: 118.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.1 KH_2PO_4, and 5.6 glucose) at room temperature. The vessel was cleaned of adhesive fat and connective tissue, care being taken not to stretch the vessel and not to damage the endothelium or smooth muscle cells. The isolated vessel was cut into rings approximately 3 mm in length.

For the recording of tension the vessel rings were mounted, between 2 L-shaped stainless steel hooks, in 5-mL organ baths filled with oxygenated Krebs-Henseleit solution at 37.8°C (pH 7.4). Each preparation was fixed, via a silk thread, to an isometric force transducer (A.D. Instruments, Castle Hill, Australia) and force was recorded via a MacLab/8 computer system (A.D. Instruments). Each vessel ring was subjected to an initial resting tension of 9.8 mN that was maintained throughout.

After an equilibration period of 45 minutes, the vascular rings were primed and tested for viability by exposing them three times to a depolarizing 40-mmol/L potassium chloride (KCl) solution. The KCl solution had the same composition as the Krebs-Henseleit solution used, except for sodium chloride, which had been partially replaced by an equimolar amount of KCl corresponding to a total concentration of 40-mmol/L KCl. After each depolarization the medium was exchanged and the vessel rings were allowed to equilibrate for 20 minutes. Between the second and the third KCL-induced vasoconstriction, the endothelial function of the vessel rings was tested by applying a precontraction with phenylephrine (1 μmol/L) followed by a single concentration (1 μmol/L) of metacholine. Vessel rings with an endothelial function ≥80% were regarded as vessels with an intact endothelium and included in the experiments. When endothelium was deliberately removed, only vessels with an endothelial function of ≤20% were regarded as endothelium-denuded and included in further experiments.

Experimental Protocol

Following the priming procedure, the vessels were subjected to either the NOS inhibitor L-NNA (100 μmol/L), the 5-HT_{1A} antagonist NAN-190 (1 μmol/L), the 5-$HT_{1A/1B}$ receptor antagonist methysergide (1 μmol/L), or to the β_2 and β_3 adrenoceptor antagonists butoxamine (50 μmol/L) and S-(−)-cyanopindolol (1 μmol/L), respectively. Other vessels were not incubated and served as controls. Thirty minutes of incubation was followed by precontraction of the vessels with the thromboxane A_2 analogue U46 (30 nmol/L) and after the contractile response had reached a plateau, cumulative concentrations of dl-nebivolol were added (100 nmol/L to 10 μmol/L). Other vessel rings were subjected to cumulative concentrations of metoprolol, a selective β_1 adrenoceptor antagonist devoid of vasodilating properties (100 nmol/L to 10 μmol/L). Furthermore, cumulative concentrations of the selective β_3-adrenoceptor agonist BRL 37344 (1 nmol/L to 10 μmol/L) were applied, in the presence and absence of L-NNA. In one series of experiments, the endothelium was mechanically removed, by gently rubbing the intima of the vessels three times gently with a cannula.

Statistical Evaluation

All responses were calculated as percentage of the vascular tone reached by preconstriction with 30-nmol/L U46. Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA), followed by the Dunnett post test for comparison versus control, or the *t* test were performed. *P* values less than 0.05 were considered to be statistically significant.

Chemicals

L-NNA (N^{ω}-nitro-L-arginine) was obtained from Alexis Biochemicals (San Diego, CA, U.S.A.). NAN-190 (1-(2-methoxyphenyl)-4-(4-phthalimidobutyl) piperazine) hydrobromide, methysergide ([8b(S)]-9,10-didehydro-N-[1-(hydroxymethyl)propyl]-1,6-dimethylergoline-8-carboxamide) maleate and BRL37344 [(*R**,*R**)-(±)-4-[2-[(2-(3-chlorophenyl)- 2-hydroxyethyl)amino]propyl] phenoxyacetic acid] sodium salt were obtained from Tocris (Ellisville, MO, U.S.A.). Phenylephrine [(R)-(−)-1-(3-hydroxyphenyl)-2-methylaminoethanol] hydrochloride, metacholine (acetyl-β-methylcholine) chloride, U46619 (9,11-dideoxy-11a, 9a-epoxymethanoprostaglandin F_{2a}), metoprolol (1-(isopropylamino)-3-(p-[β-methoxyethyl]phenoxy)-2-propanol) tartrate, butoxamine (α-(1-[t-butylamino]ethyl)-2,5-dimethoxybenzyl alcohol) hydrochloride and S-(−)-cyanopindolol (4-(3-[(1,1-dimethylethyl)amino]-2-hydroxypropyl)-1H-indole-2-carbonitrile) hemifumarate were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

dl-Nebivolol (α, α'-[iminobis(methylene)]-bis-[6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol]) was a kind gift from the Menarini Group (Florence, Italy). All compounds were dissolved in distilled water, except for nebivolol, which was dissolved in DMSO and further diluted. The organ bath concentration of DMSO never exceeded 0.002%. In this concentration, DMSO showed no pharmacological effect (data not shown).

RESULTS

In the isolated rat thoracic aorta, nebivolol, in concentrations of 3 μmol/L and higher, produced a concentration-dependent vasodilatation. In the highest concentration applied (10 μmol/L), this resulted in a vascular tone of 55.2% ± 7.8% of the applied precontraction. In similar concentrations, metoprolol did not cause vasodilatation (Fig. 1).

As shown in Figure 2, the vasodilator effect of nebivolol, in concentrations of 3 and 10 μmol/L, was significantly blocked by exposure to 100 μmol/L of the NOS inhibitor L-NNA (102.3% ± 2.3% versus 84.6% ± 4.2% and 99%.1 ± 2.2% versus 55.2% ± 7.8%, respectively; $P < 0.05$, n = 6–13). Mechanical removal of endothelium had the same effect (98.0% ± 0.9% versus 84.6% ± 4.2% and 84.62% ± 1.8% versus 55.2% ± 7.8%, respectively; $P < 0.05$, n = 6–13).

To investigate whether serotonergic pathways play a role in the observed nebivolol-induced vasorelaxation, the vessel rings were exposed to the 5-HT_{1A} specific receptor antagonist NAN-190 and to the 5-$HT_{1/2}$ receptor antagonist methysergide. Neither NAN-190 (1 μmol/L) nor methysergide (1 μmol/L) significantly affected nebivolol-induced vasorelaxation (Fig. 3).

In addition, the involvement of β-adrenergic pathways was investigated. Specific β_2-adrenoceptor blockade with butoxamine (50 μmol/L) did not influence the vasodilatory effect of nebivolol. However, the addition of 1-μmol/L S-(−)-cyanopindolol significantly affected the response to 10-μmol/L nebivolol and prevented nebivolol-induced vasorelaxation (82.0% ± 4.5% versus 55.2% ± 7.8%; $P < 0.05$, n = 6–13) (Fig. 4).

Figure 5 shows responses to the specific β_3 agonist BRL 37344, applied to confirm that β_3 adrenoceptor stimulation leads to NO-dependent vasorelaxation in the rat thoracic aorta. Indeed, BRL 37344 produced vasodilatation that was significantly blocked by L-NNA (Fig. 5). In a concentration of 1-μmol/L BRL 37344, L-NNA (100 μmol/L) increased vascular tone from 81.6% ± 5.5% to 98.8% ± 0.5% ($P < 0.05$; n = 6) and in the maximal concentration of BRL 37344 applied (10 μmol/L), from 45.1% ± 9.0% to 83.9% ± 6.2% ($P < 0.05$, n = 6). β_3 adrenoceptor blockade completely inhibited BRL 37344-induced vasorelaxation: 97.5% ± 0.8% (S-(−)-cyanopindolol) versus 81.6 ± 5.5% (control) in a concentration of 1-μmol/L BRL 37344 and 94.7% ± 1.7% (S-(−)-cyanopindolol) versus 45.1% ± 9.0% (control) in a concentration of 10-μmol/L BRL 37344 ($P < 0.05$, n = 6).

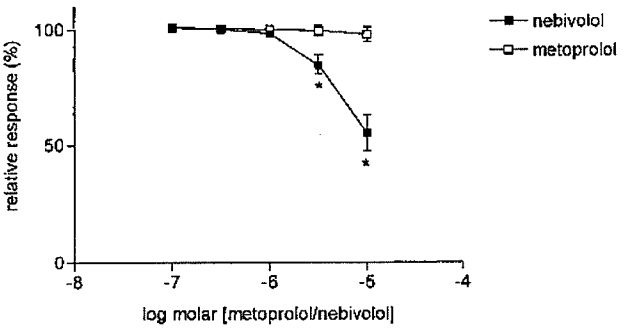

FIGURE 1. Vasodilator activity of nebivolol. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol (■) and metoprolol (□). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus metoprolol.

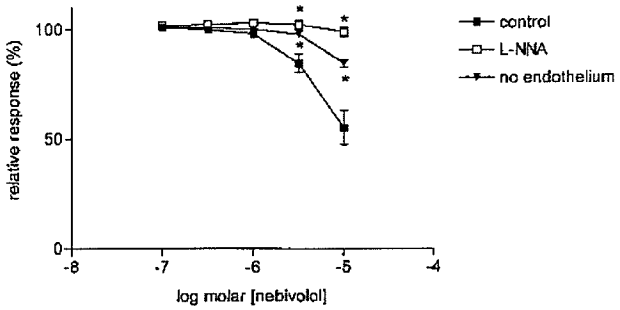

FIGURE 2. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol under control conditions (■), after incubation with 100-μmol/L L-NNA (□) and after mechanical removal of the endothelium (▼). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus control.

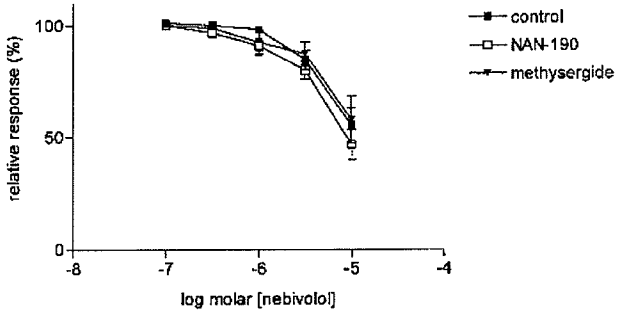

FIGURE 3. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol under control conditions (■), after incubation with 1-μmol/L NAN-190 (□), and after incubation with 1-μmol/L methysergide (▼). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus control.

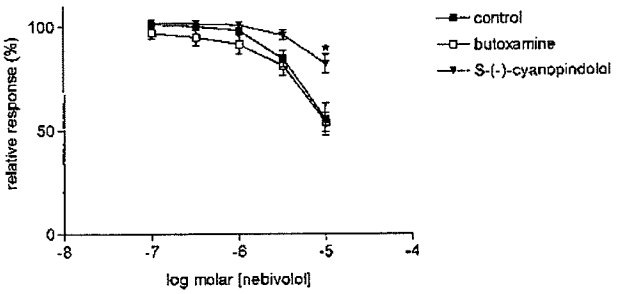

FIGURE 4. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of nebivolol under control conditions (■), after incubation with 50-μmol/L butoxamine (□), and after incubation with 1-μmol/L S-(−)-cyanopindolol (▼). Data are expressed as mean ± SEM (n = 6–13). *$P < 0.05$ versus control.

DISCUSSION

In the present study, we investigated the vasodilating properties of nebivolol in the rat aorta. Nebivolol produced endothelium/NO-dependent vasodilatation in similar concentrations as applied in previous studies.[3,8] In the same concentration range, metoprolol, a β_1-adrenoceptor antagonist without NO-releasing activity, did not cause vasorelaxation.

In an organ bath model similar to ours, Altwegg et al[9] did not observe nebivolol-induced vasorelaxation in the aorta. However, our results are in accordance with those of Kakoki et al,[8] who also reported endothelium-dependent vasodilating effects of nebivolol in rat aortic rings. In contrast to our results, in their study, the 5-HT_1 blocker NAN-190 (100 nmol/L) was able to inhibit vasorelaxation by nebivolol. It is worth mentioning that, in experiments performed in our model, NAN-190, in a concentration as low as 1 nmol/L, attenuated phenylephrine-induced vasoconstriction and in higher concentrations (10 and 100 nmol/L) even abolished the α_1-adrenoceptor–mediated contractile response completely (data not shown). These findings imply that NAN-190 cannot be regarded as specific for 5-HT_1 receptors: it blocks α_1-adrenoceptors and possibly activates or deactivates other adrenergic receptors. The response to U46, the thromboxane A_2-analogue we applied as a preconstrictor agent, was not affected by incubation with NAN-190. In these experiments, NAN-190 did not influence nebivolol-induced vasodilatation. However, the specificity of NAN-190 remains questionable and therefore results obtained with the 5-HT_1 and 5-HT_2 blocker methysergide, that in the same concentration range did not affect phenylephrine-induced vasoconstriction, appear to be more conclusive. Previous studies performed with methysergide in the canine coronary artery showed no serotonergic involvement in nebivolol-induced vasorelaxation.[3] We demonstrated that methysergide does not affect nebivolol-induced vasorelaxation in the rat aorta, suggesting that the NO-dependent dilating properties of nebivolol are not mediated by a serotonergic pathway.

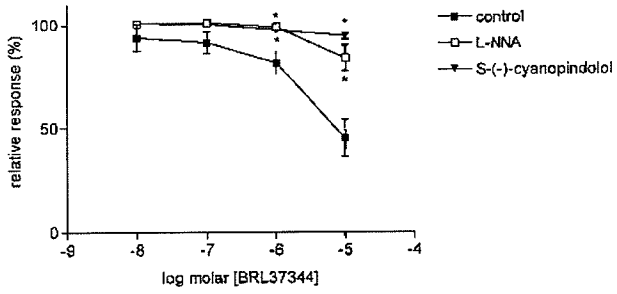

FIGURE 5. Vasodilator activity of BRL 37344. Relative vascular tone of isolated rat aortic rings in response to cumulative concentrations of BRL 37344 under control conditions (■), after incubation with 100-μmol/L L-NNA (□), and after incubation with 1 μM S-(−)-cyanopindolol (▼). Data are expressed as mean ± SEM (n = 6). *$P < 0.05$ versus control.

We therefore hypothesized that nebivolol might exert its NO-mediated vasodilating action through a β_2- or β_3-adrenoceptor–mediated pathway. To investigate both pathways, the vessel rings were subjected to the β_2-adrenoceptor antagonist butoxamine and the β_3-adrenoceptor antagonist S-(−)-cyanopindolol. S-(−)-cyanopindolol significantly attenuated the vasorelaxation by nebivolol. β_2 blockade with butoxamine did not affect the concentration response curve. These results suggest that in the rat aorta, NO-dependent vasorelaxation by nebivolol may be mediated by β_3 receptor stimulation. This presumption is in accordance with the findings of Gosgnach et al,[11] who analyzed the different signaling pathways implicated in the response of human umbilical vein endothelial cells to nebivolol. They suggest that nebivolol possesses β_3-adrenoceptor agonistic properties that seem to be responsible for its endothelium-dependent vasodilating activity.

Most studies on the β_3-adrenoceptor subtype have focused on its control of lipolysis in adipose tissues. However, more recent studies have investigated the involvement of β_3-adrenoceptors in the physiological control of cardiac and vascular contractility. These studies have produced pharmacological and molecular evidence that supports the functional role of β_3-adrenoceptors in cardiovascular tissues of various species.[12] Furthermore, they have demonstrated that β_3 receptor activation indeed causes relaxation both in cardiac and in vascular tissue via NOS activation leading to release of NO.[13]

To confirm that β_3 adrenoceptor activation causes endothelium-dependent vasorelaxation in our model, we exposed aortic rings to cumulative concentrations of BRL 37344, a specific β_3 adrenoceptor agonist. Indeed, BRL 37344 produced concentration-dependent relaxations that were inhibited by L-NNA as well as by β_3-adrenoceptor blockade with S-(−)-cyanopindolol.

CONCLUSION

Based on the data presented in this functional study, we conclude that, in the rat aorta, nebivolol-induced NO-

dependent vasorelaxation is, at least in part, caused by a β_3-agonistic effect.

REFERENCES

1. Pessina AC. Metabolic effects and safety profile of nebivolol. *J Cardiovasc Pharmacol.* 2001;38(suppl 3):S33–S35.
2. Ritter JM. Nebivolol: endothelium-mediated vasodilating effect. *J Cardiovasc Pharmacol.* 2001;38(suppl 3):S13–S16.
3. Gao YS, Nagao T, Bond RA, et al. Nebivolol induces endothelium-dependent relaxations of canine coronary arteries. *J Cardiovasc Pharmacol.* 1991;17:964–969.
4. Bowman AJ, Chen CP, Ford GA. Nitric oxide mediated venodilator effects of nebivolol. *Br J Clin Pharmacol.* 1994;38:199–204.
5. Cockcroft JR, Chowienczyk PJ, Brett SE, et al. Nebivolol vasodilates human forearm vasculature: evidence for an L-arginine/NO-dependent mechanism. *J Pharmacol Exp Ther.* 1995;274:1067–1071.
6. Dawes M, Brett SE, Chowienczyk PJ, et al. The vasodilator action of nebivolol in forearm vasculature of subjects with essential hypertension. *Br J Clin Pharmacol.* 1999;48:460–463.
7. Van Nueten L, De Cree J. Nebivolol: comparison of the effects of dl-nebivolol, d-nebivolol, l-nebivolol, atenolol, and placebo on exercise-induced increases in heart rate and systolic blood pressure. *Cardiovasc Drugs Ther.* 1998;12:339–344.
8. Kakoki M, Hirata Y, Hayakawa H, et al. Effects of vasodilatory beta-adrenoceptor antagonists on endothelium- derived nitric oxide release in rat kidney. *Hypertension.* 1999;33:467–471.
9. Altwegg LA, d'Uscio LV, Barandier C, et al. Nebivolol induces NO-mediated relaxations of rat small mesenteric but not of large elastic arteries. *J Cardiovasc Pharmacol.* 2000;36:316–320.
10. Broeders MA, Doevendans PA, Bekkers BC, et al. Nebivolol: a third-generation beta-blocker that augments vascular nitric oxide release: endothelial beta(2)-adrenergic receptor-mediated nitric oxide production. *Circulation.* 2000;102:677–684.
11. Gosgnach W, Boixel C, Nevo N, et al. Nebivolol induces calcium-independent signaling in endothelial cells by a possible beta-adrenergic pathway. *J Cardiovasc Pharmacol.* 2001;38:191–199.
12. Gauthier C, Langin D, Balligand JL. Beta3-adrenoceptors in the cardiovascular system. *Trends Pharmacol Sci.* 2000;21:426–431.
13. Trochu JN, Leblais V, Rautureau Y, et al. Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta. *Br J Pharmacol.* 1999;128:69–76.